REMARKS

Applicants respectfully request reconsideration of the patent application.

I. Status of Claims

1. Claims 1-2, 13, 16-17, 51, 53, 81-89 are currently pending in the application.

2. Applicants acknowledge that claims 2, 51 and 53 are noted as being allowed.

3. Claims 16, 81 and 89 have been amended in this response in order to advance prosecution of the pending application. The amendments to these claims have been made without the introduction of new matter. Specifically, in Claim 89 Applicants have amended the % of amino acid identity from 71% to 95% as consistent with the U.S. Patent Office Written Description Guidelines at www.uspto.gov/web/offices/pac/writtendesc.pdf

4. Claims 83-88 have been cancelled without waiver or prejudice in order to advance prosecution of the present application.

5. Applicants reserve the right to pursue the subject matter of any current or previously withdrawn or cancelled claims in one or more continuing applications.

II. Withdrawn Claims

6. Claims 81-86 were withdrawn from consideration as being directed to a non-elected invention. Applicants respectfully traverse the withdrawal of these claims.

Claim 81 has been amended to incorporate the subject matter of Claim 82, such that presently amended claim 81 fully complies with Applicants' election to prosecute the claims of Group I, being drawn to a nucleic acid encoding VDRRg, expression vector, host cell, and method of producing the polypeptide. Support for claim 81 is found in the specification at page 4, line 30 to page 5, line 8 where it is stated that the recombinant polypeptide may be truncated forms of the full-length polypeptide.

Claims 82-86 have been cancelled without waiver or prejudice to advance the prosecution of the present application.

In view of the amendment to Claim 81, Applicants request the Examiner to rejoin this claim with the remaining pending claims of the present application.

III. Claim Rejections-35 U.S.C. §112

7. Claims 16 and 87-89 were rejected under 35 U.S.C. §112, second paragraph as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicants regard as their invention. Applicants respectfully traverse this rejection.

Claim 16 has been amended to moot this rejection by removal of the confusing terms.

Claims 87 and 88 have been cancelled without waiver or prejudice for purposes of advancing the prosecution of the present application.

Claim 89 has been amended to remove the term "NR1I2" in order to moot this rejection. Applicants note that the term "NR1I2" is not the name of a compound. Applicants submit that "NR1I2" is a term which is recognized by those of skill in the art to define the nuclear receptor as presently claimed. Applicants submit as Exhibit 1 herewith a printout from NCBI website noting the currently accepted nomenclature for the claimed nuclear receptor. Applicants request the Examiner to reconsider Applicants "NR1I2" nomenclature change. See *Schering Corp. v. Amgen Inc.*, 55 USPQ2d 1650, 1654 (CA FC 2004). Further, Applicants request that the Examiner add the term "NR1I2" back to claim 89 by an Examiner's Amendment.

In view of the above amendments, Applicants request withdrawal of the rejection and reconsideration of the claims 16 and 89.

8. Claims 87-89 were rejected under 37 U.S.C. §112, first paragraph as failing to comply with the written description requirement. Applicants respectfully traverse this rejection.

Claims 87 and 88 have been cancelled by this amendment without waiver or prejudice.

Claim 89 has been amended to specify "95% sequence identity" thus being consistent with the written description guidelines of the United States Patent and Trademark Office. It is submitted that this amended percentage sequence identity moots the written description rejection.

The U.S. Patent Office has provided guidelines for determining whether the written description requirement is satisfied where a claim recites a "Product by Function" *See* "Revised Interim Written Description Guidelines Training Materials," available at http://www.uspto.gov/web/menu/written.pdf

In particular, Example 14 therein, provides circumstances under which a claim to "[a] protein having SEQ ID NO:[#] and variants thereof that are **at least 95% identical** to SEQ ID NO:[#] and catalyze the reaction A→ B" is fully supported by the specification. *See Id.* at page 53 (emphasis added).

It is submitted that claim 89, as amended, now meets the written description requirements of the U.S. Patent Office "Guidelines".

Applicants request that the Examiner reconsider and withdraw the rejection under 35 U.S.C. §112, first paragraph as failing to comply with the written description requirement.

9. Claims 87-88 were rejected under 35 U.S.C. §112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed had possession of the claimed invention. Applicants respectfully traverse this rejection.

Claims 87 and 88 have been cancelled without waiver or prejudice in order to advance the prosecution of the pending application. Thus, this rejection has been mooted.

Applicants reserve their right to file a continuing or divisional application(s) to pursue the subject matter contained in these cancelled claims.

10. Claims 87-89 were rejected under 35 U.S.C. § 112, first paragraph, because the specification, while being enabling for isolated cells transfected or transformed with an expression vector does not reasonably provide enablement for an isolated cell comprising a nucleic acid which is not contained in a vector. Applicants respectfully traverse this rejection.

Claims 87 and 88 have been cancelled without waiver or prejudice by this amendment.

Claim 89, as amended, does not recite the terms "isolated cell". Thus it is submitted that a *prima facie* rejection under 35 U.S.C. §112, first paragraph, has not been established on the basis set forth by the Examiner.

Applicants request that the Examiner reconsider and withdraw the rejection under 35 U.S.C. §112, first paragraph, for alleged lack of enablement.

IV. Priority Rejection

11. Claims 1-2, 13, 16-17, 51, 53 and 87-89 were rejected under 35 U.S.C. §112 on the basis that these claims did not receive priority to Applicants' parent applications, U.S. Patent Application No. 60/067,373 and SE-9703745-1 on the basis that these applications did not disclose the function of the orphan receptor. Applicants respectfully traverse this rejection.

Claims 2, 51 and 53 have been noted as being allowed. Clarification is requested by the Examiner.

The test for determining whether Applicants are entitled to the priority of their earlier filed parent applications was set forth in *Noelle v. Lederman,* 69 USPQ 2d 1058, 1513 (CA FC 2004)(Copy enclosed). The test as set forth provides:

> "[t]he test to determine if an application is to receive the benefit of an earlier filed application is whether a person of ordinary skill in the art would recognize that the applicant possessed what is claimed in the later filed application as of the filing date of the earlier filed application. An earlier application that describes later-claimed genetic material only by a statement of function or result may be insufficient to meet the written description requirement. *See Regents*, 119 F.3d at 1566. This court has held that a description of DNA " 'requires a precise definition, such as by structure, formula, chemical name, or physical properties', not a mere wish or plan for obtaining the claimed chemical invention." *Id.* (quoting *Fiers v. Revel*, 984 F.2d 1164, 1170 [25 USPQ 2d 1601] (Fed. Cir. 1993)). Therefore, this court has held that statements in the specification describing the functional characteristics of a DNA molecule or methods of its isolation do not adequately describe a particular claimed DNA sequence. Instead "an adequate written description of DNA requires more than a mere statement that it is part of the invention and reference to a potential method for isolating it; what is required is a

> description of the DNA itself." *Id.* At 1566-67 (quoting *Fiers*, 984 F.2d at 1171)."

It is submitted that as Applicants' prior parent applications, U.S. Patent Application No. 60/067,373 and SE-9703745-1 adequately set forth the nucleotide and amino acid sequences as presently claimed, as well as teachings that these sequences are nuclear receptors, see, for example, page 7, line 20 to page 8, line 2 of SE-9703745-1. In further support of Applicants teachings at page 7, line 20 to page 8, line 2 Applicants submit herewith Exhibits 2-3.

Exhibit 2 is the paper of Pascussi, J.M. et al., (2003) *Biochimica et Biophysica Acta,* 1619: 243-253, wherein the Abstract states:

> "[i]ndeed, this adaptive system appears now as a tangle of networks, where receptors share partners, ligands, DNA response elements and target genes."

Exhibit 3 is the paper of Ryutaro, A. et al., (2005) *Journal of Lipid Research,* 46:46-57, wherein the Abstract states:

> "[r]ecently VDR was found to respond to bile acids as well as other nuclear receptors, farnesoid X receptor(FXR) and pregane X receptor (PXR)"

Applicants note that the PXR acronym noted in Exhibit 3 is a gene aliases/synonym of the presently claimed NR1I2 nuclear receptor as noted in Exhibit 1 and as referred to in Applicants' specification as "VDRR".

Thus, Applicants are entitled to the priority of these earlier filed parent applications.

Applicants request that the Examiner reconsider and withdraw the objection to Applicants' claim for the domestic priority under 35 U.S.C. §120 for the prior patent applications, U.S. Patent Application No. 60/067,373 and S.E. 9703745-1.

V. Claim Rejections – 35 U.S.C. §102

Claims 1-2, 13, 16-17 and 87-89 were rejected under 35 U.S.C. §102(e) as being anticipated by Evans et al. (U.S. 6,756,491). Applicants respectfully traverse this rejection.

Claim 2 has been noted as being allowed. Clarification is requested by the Examiner.

It is respectfully submitted that a *prima facie* rejection under 35 U.S.C. §102(e) has not been properly established. As argued above, the claims of the present invention are entitled to the priority of their earlier filed parent applications, U.S. Patent Application No. 60/067,373 and SE-9703745-1. Thus, the rejection under 35 U.S.C. §102(e) has been mooted.

In addition, it is submitted that the holding of *In re Wallach*, 71 USPQ 2d 1939, 1943 (CA FC 2004) (Copy enclosed) is independently applicable to moot the pending anticipation rejection. Applicants' parent applications provided the full-length amino acid sequence for the claimed nuclear receptor at Fig. 4 and are thus are entitled to their earlier priority date than that of the Evans reference; and to the scope of the claims as presently pending.

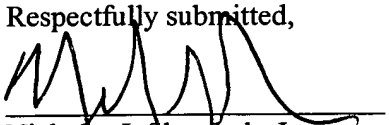
Applicants request that the Examiner reconsider and withdraw the rejection under 35 U.S.C. §102(e), as being anticipated by Evans et al.

VI. Conclusion

Applicants submit that all the grounds for rejection of the pending claims have now been overcome and that all the claims are now in condition for allowance, which action is respectfully requested.

In the event that the Examiner wishes to discuss any aspect of this communication, please contact the undersigned Attorney at the telephone number provided below.

Respectfully submitted,



Nicholas I. Slepchuk, Jr.
Attorney for the Applicants
Reg. No. 32,174

Date: August 25, 2005

Pfizer Inc.
Patent Department
MS8260-1611
Eastern Point Road
Groton, CT 06340
(617) 551-3223

EXHIBIT 1

1: NR1I2 nuclear receptor subfamily 1, group I, member 2 [*Homo sapiens*]

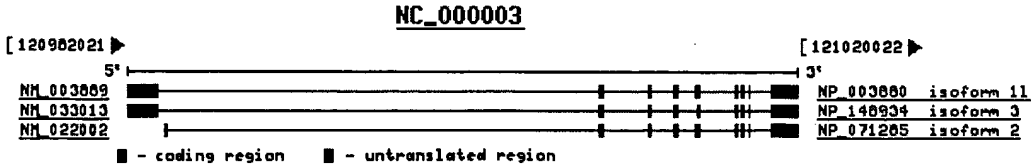
GeneID: 8856 Locus tag: HGNC:7968; MIM: 603065 updated 02-Aug-2005

Summary

Official Symbol: NR1I2 **and Name:** nuclear receptor subfamily 1, group I, member 2 **provided by** HUGO Gene Nomenclature Committee
Gene type: protein coding
Gene name: NR1I2
Gene description: nuclear receptor subfamily 1, group I, member 2
RefSeq status: Reviewed
Organism: *Homo sapiens*
Lineage: *Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Mammalia; Eutheria; Euarchontoglires; Primates; Catarrhini; Hominidae; Homo*
Gene aliases: BXR; PAR; PRR; PXR; SAR; SXR; ONR1; PAR1; PAR2; PARq
Summary: The gene product belongs to the nuclear receptor superfamily, members of which are transcription factors characterized by a ligand-binding domain and a DNA-binding domain. The encoded protein is a transcriptional regulator of the cytochrome P450 gene CYP3A4, binding to the response element of the CYP3A4 promoter as a heterodimer with the 9-cis retinoic acid receptor RXR. It is activated by a range of compounds that induce CYP3A4, including dexamethasone and rifampicin. The gene product contains a zinc finger domain. Three alternatively spliced transcripts that encode different isoforms have been described, one of which encodes two products through the use of alternative translation initiation codons. Additional transcript variants derived from alternative promoter usage, alternative splicing, and/or alternative polyadenylation exist, but they have not been fully described.

Transcripts and products

RefSeq below

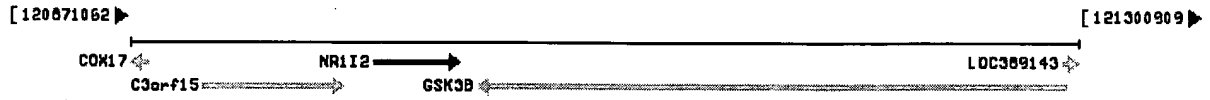


Genomic context

See NR1I2 in MapViewer

chromosome: 3; **Location:** 3q12-q13.3

Entre
Table
Summary
Transcripts
Genomic c
Bibliograph
General ge
General pr
Reference
Related Se
Additional L
Conserved
GEO Profil
HomoloGe
Map Viewe
Nucleotide
OMIM
Full text in
Probe
Protein
PubMed
PubMed (G
SNP
SNP: Geno
SNP: Gene
Taxonomy
UniSTS
AceView
Ensembl
Evidence V
GDB
HGNC
KEGG
MGC
ModelMake
PharmGKB
UCSC
UniGene
LinkOut
Entr
F
Sul



Bibliography Gene References into Function (GeneRIF): **Submit**

PubMed links

GeneRIFs:

1. pxr polymorphism is associated with decreased expression of MDR1 mRNA in intestinal villi — PubMed
2. xenobiotics and drugs can modulate 25-hydroxyvitamin D(3)-24-hydroxylase gene expression and alter vitamin D(3) hormonal activity and calcium homeostasis through the activation of PXR — PubMed
3. PXR-mediated gene regulation is affected by its DNA binding site — PubMed
4. expressed approximately 250-fold lower in peripheral blood mononuclear cells than in liver, and significantly correlated to MDR1 mRNA — PubMed
5. ligand-activated PXR interferes with HNF-4 signaling by targeting the common coactivator PGC-1, which underlies physiologically relevant inhibitory cross-talk between drug metabolism and cholesterol/glucose metabolism — PubMed
6. pregnane X receptor (PXR) and constitutively activated receptor (CAR) mediate induction of CYP3A5 in human liver and intestine — PubMed
7. pregnane X receptor is a major determinant of CYP2B6-inducible expression — PubMed
8. individual variation in pregnane X receptor expression may account for differential expression of some UDP-glucuronosyltransferase isoforms between subjects — PubMed
9. The present data indicate that SXR is a key system to induce, maintain and reverse a cisplatin-resistant phenotype in endometrial cancer cells. — PubMed
10. steroid and xenobiotic receptor(SXR) has a novel role as a mediator of bone homeostasis in addition to its role as a xenobiotic sensor — PubMed
11. 2.0A crystal structure of the human PXR ligand-binding domain (LBD) in complex with the cholesterol-lowering compound SR12813 and a 25 amino acid residue fragment of the human steroid receptor coactivator-1 (SRC-1) containing one LXXLL motif — PubMed
12. Pregnane X receptor mRNA expression levels are compared in a panel of 12 individual human liver samples; a 27-fold variability in PXR mRNA expression is found. — PubMed
13. results provide evidence that the nuclear import of SXR is mediated by bipartite type of nuclear localization signal, which is recognized by three groups of importin adaptors for targeting the nuclear rim — PubMed
14. control of steroid, heme, and carcinogen metabolism by this protein in transgenic mice — PubMed
15. human PXR requires a specific agonist different from that required in mice to induce cyp3A expression — PubMed
16. mediates the proliferative action of mast-cell tryptase: possible relevance to human fibrotic disorders — PubMed
17. alternative splicings for hPXR may largely contribute to the interindividual variability in CYP3A4 and P-glycoprotein induction — PubMed
18. Role of SXR in xenobiotic inhibition of CYP3A4 promotor activity — PubMed
19. promiscuous nuclear receptor that responds to a wide variety of drugs, xenobiotics and endogenous compounds, and plays a critical role in mediating — PubMed

drug-drug interactions in humans

General gene information

GeneOntology

Provided by GOA

Function	Evidence	
metal ion binding	IEA	
steroid hormone receptor activity	IEA	
transcription coactivator activity	TAS	PubMed
transcription factor activity	IEA	
Process		
regulation of transcription, DNA-dependent	IEA	
signal transduction	TAS	PubMed
steroid metabolism	TAS	PubMed
transcription	IEA	
xenobiotic metabolism	TAS	PubMed
Component		
nucleus	IEA	

Homology:
Mouse, Rat
Map Viewer

Markers (Sequence Tagged Sites/STS)
SHGC-56597 (e-PCR)
Alternate name RH100616
Alternate name RH81342
NR1I3_2131 (e-PCR)

General protein information

Names: pregnane X receptor
steroid and xenobiotic receptor

NCBI Reference Sequences (RefSeq)

mRNA Sequence NM_003889
Transcriptional Variant
Transcript Variant: This variant (1) has a different 5' UTR than variant 2. It encodes two products (isoforms 11 and 1s) through the use of alternative translation initiation codons, which are in the same reading frame. A non-AUG translation initiation codon is used by isoform 11. Isoforms 11 and 1s lack an amino-terminal 39 and 94 amino acids, respectively, compared to isoform 2.
Source Sequence AJ009936
Product NP_003880 pregnane X receptor isoform 11
Conserved Domains (2) summary
smart00399: ZnF_C4; c4 zinc finger in nuclear hormone receptors
Location: 38 - 110 Blast Score: 270
smart00430: HOLI; Ligand binding domain of hormone receptors
Location: 245 - 404 Blast Score: 231

mRNA Sequence NM_022002

Transcriptional Variant

Transcript Variant: This variant (2) has a different 5' UTR than other variants. It encodes a single full-length product (isoform 2) with an amino-terminal extension not found in other isoforms.

Source Sequence AF084644

Product NP_071285 pregnane X receptor isoform 2

Consensus CDS (CCDS) CCDS2995.1

Conserved Domains (2) summary

smart00399: ZnF_C4; c4 zinc finger in nuclear hormone receptors
Location: 77 - 149 Blast Score: 274

smart00430: HOLI; Ligand binding domain of hormone receptors
Location: 284 - 443 Blast Score: 236

mRNA Sequence NM_033013

Transcriptional Variant

Transcript Variant: This variant (3) has a different 5' UTR than variant 2. It encodes an isoform (3) that lacks 39 amino-terminal and 37 internal amino acids compared to isoform 2; the reading frame is maintained. Isoform 3 uses a non-AUG translation initiation codon.

Source Sequence AJ009937

Product NP_148934 pregnane X receptor isoform 3

Conserved Domains (2) summary

smart00399: ZnF_C4; c4 zinc finger in nuclear hormone receptors
Location: 38 - 110 Blast Score: 270

smart00430: HOLI; Ligand binding domain of hormone receptors
Location: 208 - 367 Blast Score: 228

Related Sequences

Nucleotide		Protein
Genomic	AF364606	AAK38720
		AAK38721
		AAK38722
Genomic	AY007189	AAG23345
mRNA	AF061056	AAD05436
mRNA	AF084644	AAC64557
mRNA	AF084645	AAC64558
mRNA	AJ009936	CAB55489
		CAB55490
		CAB55491
mRNA	AJ009937	CAB55492
		CAB55493
		CAB55494
mRNA	AK122990	None
mRNA	AK223456	BAD97176
mRNA	AY091855	AAM26736
mRNA	BC017304	AAH17304
	None	O75469

Additional Links

UniGene Hs.7303
MIM 603065
PharmGKB PA378

Display Graphics Show 5 Send to

EXHIBIT 2

ELSEVIER

Biochimica et Biophysica Acta 1619 (2003) 243–253

BIOCHIMICA ET BIOPHYSICA ACTA
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Review

The expression of CYP2B6, CYP2C9 and CYP3A4 genes: a tangle of networks of nuclear and steroid receptors

J.M. Pascussi*, S. Gerbal-Chaloin, L. Drocourt, P. Maurel, M.J. Vilarem

Institut National de la Santé et de la Recherche Médicale U128, IFR 24, Centre National de la Recherche Scientifique, 1919 route de Mende, 34293 Montpellier 05, France

Received 25 March 2002; received in revised form 9 July 2002; accepted 10 October 2002

Abstract

Numerous chemicals increase the metabolic capability of organisms by their ability to activate genes encoding various xenochemical-metabolizing enzymes, such as cytochromes P450 (CYPs), transferases and transporters. For example, natural and synthetic glucocorticoids (agonists and antagonists) as well as other clinically important drugs induce the hepatic CYP2B, CYP2C and CYP3A subfamilies in man, and these inductions might lead to clinically important drug–drug interactions. Only recently, the key cellular receptors that mediate such inductions have been identified. They include nuclear receptors, such as the constitutive androstane receptor (CAR, NR1I3), the retinoid X receptor (RXR, NR2B1), the pregnane X receptor (PXR, NR1I2), and the vitamin D receptor (VDR, NR1I1) and steroid receptors such as the glucocorticoid receptor (GR, NR3C1). There is a wide promiscuity of these receptors in the induction of CYPs in response to xenobiotics. Indeed, this adaptive system appears now as a tangle of networks, where receptors share partners, ligands, DNA response elements and target genes. Moreover, they influence mutually their relative expression. This review is focused on these different pathways controlling human CYP2B6, CYP2C9 and CYP3A4 gene expression, and the cross-talk between these pathways.

Keywords: Nuclear receptor; Cytochrome P450; Gene expression regulation; Xenobiotic

1. Introduction

The cytochrome P450 (CYP) proteins form a superfamily of heme-containing enzymes involved in the oxidative metabolism of lipophilic compounds, including steroids, fatty acids, retinoids, bile acids and foreign chemicals such as drugs and other xenobiotics [1]. Human beings have 17 known CYP gene families, among which only the first three, CYP1, CYP2 and CYP3, are involved in the metabolism of drugs and xenobiotics. CYP-mediated detoxication is a key defense mechanism whereby organisms protect themselves from the potentially harmful effects of foreign (hydrophobic) chemicals to which they are exposed. The xenobiotics are converted to more hydrophilic compounds, which are more easily conjugated and excreted. Apart from being involved in drug metabolism, these CYPs also play an important role in cholesterol biosynthesis, vitamin D metabolism, bile acid metabolism and biosynthesis catabolism of steroids [2].

Transcriptional expression of CYP genes can be modulated both by endogenous compounds and structurally diverse xenobiotics, which may or may not be substrates of the corresponding enzymes. Of particular interest is the tissue-selective transcriptional induction of CYP genes in response to xenobiotic inducers. For example, natural and synthetic glucocorticoids (agonists and antagonists), as well as other clinically important drugs, induce CYP2B, CYP2C and CYP3A subfamilies in the liver of rodents and humans in vivo and in cultured hepatocytes from these species [3]. CYP2B6 has long been thought to play a minor role in human drug metabolism and has therefore received little attention. However, several recent findings have generated an increased interest in this isoenzyme: identification of ethnic differences in its expression [4], identification of new substrates for CYP2B6 and a possible shared specificity with CYP3A4 [5], and the suggestion that its transcriptional activation is regulated by mechanisms similar to those affecting CYP3A4 [6–9]. CYP2C9 is a member of the

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E-mail address: pascussi@montp.inserm.fr (J.M. Pascussi).

0304-4165/02/$ - see front matter
PII: S0304-4165(02)00483-X

CYP2C subfamily, which includes in humans at least three other members, i.e. CYP2C8, CYP2C18 and CYP2C19. CYP2C9 is involved in the metabolism of numerous substrates including phenytoin, tolbutamide, torsemide, S-warfarin and numerous nonsteroidal anti-inflammatory drugs [10]. The CYP3A subfamily represents the most abundant cytochromes P450 in adult human liver, comprising approximately 30% of the total content. The human CYP3A family comprises four enzymes that show variable levels of expression in the population, CYP3A4, CYP3A43, CYP3A5 and CYP3A7. Among them, the CYP3A4 isoform is the most prevalent in adults. It has been estimated that about 50% of currently marketed drugs are metabolized by CYP3A4 [11]. The substrates for this enzyme include drugs such as quinidine, nifedipine, diltiazem, lidocaine, lovastatin, erythromycin, cyclosporin, triazolam, midazolam and endogenous substances, including testosterone, progesterone, androstenediol and bile acids [12]. CYP3A4 also activates procarcinogens, including aflatoxinB1, PAHs, NNK and 6-aminochrysene [12]. CYP3A4 is induced in human hepatocytes by rifampicin [13,14], dexamethasone [14–16]; calcium channel modulators such as nifedipine and derivatives [17], phenobarbital [14,18–20] and vitamin D [21–23] among others.

In humans these inductions lead to clinically important drug–drug interactions when these substances are administered concurrently with medications which are normally metabolized by these CYPs. Understanding the molecular events leading to CYP2 and CYP3A induction, and determining whether these genes share identical regulatory mechanisms, should ultimately lead to better models for the screening and prediction of drug interactions. Recently, several members of the nuclear hormone receptor superfamily such as constitutive androstane receptor (CAR) [24], pregnane X receptor (PXR) [25–28], vitamin D receptor (VDR) [21,23] and retinoid X receptor alpha (RXRα) [29,30] as well as the glucocorticoid receptor (GR) [15,31, 32] have been shown to be responsible for and/or involved in endobiotic- and xenobiotic-mediated induction of CYP2/3A genes.

Nuclear hormone receptors (NHRs) are a large family of structurally related, ligand-activated transcriptional regulators that include more than 50 distinct proteins [33]. The general mechanism for NHR-dependent transcriptional activation initially involves an interaction between the receptor and a specific ligand [34]. Ligand binding induces a conformational change within the receptor that facilitates binding of coactivator proteins which eventually modulates the transcriptional activity of the target gene [35]. NHRs are categorized into three subclasses. Class I contains receptors for steroid hormones such as progesterone, androgens, mineralocorticoids, estrogens and glucocorticoids. In the absence of ligand these receptors are associated with molecular chaperones, such as the heat shock proteins (HSPs). After binding the hormone, class I receptors undergo a series of structural and functional changes including conformational change, dissociation from HSPs, homodimerisation, phosphorylation, migration into the nucleus, binding to DNA at specific hormone response elements located in the promoter of the target genes, and interaction with coactivators leading to the recruitment of the basal transcription machinery which eventually results in a stable preinitiation complex [36]. NHRs of Class II contain receptors for thyroid hormone, vitamin D3, 9-*cis*-retinoid and all-*trans*-retinoid receptors. These receptors are present in the nucleus as heterodimers with RXR bound to their response element in the promoter of target genes, in the absence of ligand. Upon ligand binding, they undergo a conformational change that results in the release of corepressors and recruitment of coactivators. This leads to the recruitment of the basal transcription machinery and formation of a stable preinitiation complex. NHRs of Class III contain orphan receptors whose cognate ligands have not yet been identified and for which only minimal information is available concerning the mechanism of transactivation [34].

2. The PXR

Previous work in the rat from the group of Guzelian suggested that induction of CYP3A by glucocorticoids, and paradoxically by antiglucocorticoids, is dependent on a nonclassical glucocorticoid-mediated induction process [37]. Indeed, these studies revealed an atypical profile of CYP3A23 induction as compared to the classical glucocorticoid-mediated induction of known GR-dependent genes [14,38]. For example, induction of CYP3A23 by dexamethasone requires a concentration 100 times that necessary for maximum induction of TAT (thyrosine amino transferase) gene. Further, the potency of various glucocorticoids for inducing CYP3A23 does not correlate with their potency for inducing TAT. Although computer analysis of approximately 1 kb of the human CYP3A4 proximal promoter revealed the presence of putative binding sites for the estrogen receptor, COUP-TF, HNF4, HNF5 and Oct-1 [39], no consensus binding site for the GR was identified within this region, despite the fact that CYP3A4 is transcriptionally activated by glucocorticoids [32,40]. This favored the notion that activation of this gene by glucocorticoids might proceed via a nonconsensus glucocorticoid-response unit, as suggested for the rat CYP3A23 [41,42]. Indeed, the responsive elements identified in rat and human CYP3A were identical to the consensus binding sites for nuclear receptors (AGGTCA), and the discovery of the PXR (NR1I2) and elucidation of its role unambiguously confirmed this observation [27].

In man, PXR mediates xenobiotic-mediated induction of CYP3A4 [25,26,28], CYP3A7 [43,44], CYP2B6 [6], as well as MDR1 [45], BSEP [46] and MRP2 [47]. Recent results suggest that CYP2C8 and CYP2C9 are also regulated by PXR [31,48,49]. Thus, PXR coordinately regulates genes involved in the metabolism and elimination of poten-

tially harmful xenobiotics. PXR interacts with its cognate response elements in the 5′-flanking region of target genes by forming a heterodimer with the 9-*cis*-retinoid acid receptor alpha (RXRα, NR2B1). In rat CYP3A23 and human CYP3A4, these elements consist of two copies of the AG(G/T)TCA hexanucleotide organized as a direct repeat with a three-nucleotide spacer (DR3), and an everted repeat separated by 6 bp (ER6), respectively. Some of these elements are also recognized by CAR [50] and VDR [21,23] after heterodimerization with RXR. These observations suggest that these receptors are capable of regulating a same series of genes through the same *cis*-acting elements, and that cross-talk between these signalling pathways is an important factor in mounting an appropriate response to a xenobiotic challenge (Fig. 1, Table 1).

PXR, like its primary gene target CYP3A4, is mainly expressed in the liver, small intestine and colon [28]. This receptor binds, generally with low affinity, a wide variety of structurally diverse exogenous and endogenous chemicals, including drugs such as rifampicin, phenobarbital, nifedipine and other calcium channel blockers, clotrimazole, mifepristone, metyrapone [17,25,26,28,32,51–53], steroid hormones and metabolites such as progesterone, estrogens, corticosterone, 5β-pregnanes and androstenol, and dietary compounds such as coumestrol and hyperforin [51]. The latter compound is a constituent of St. John's wort, a herbal remedy for depression, which appears to be the most potent PXR activator with an EC_{50} of 23 nM. An intriguing aspect concerned the ability of PXR to be activated by glucocorticoids and notably dexamethasone. This compound is a potent inducer of CYP3A in rat and mouse and a moderate inducer in human. It had been initially described as a weak activator of mouse and human PXRs [27,28]. However, Bertilsson et al. [25] found no activation of human PXR by dexamethasone and concluded that an additional mechanism was responsible for dexamethasone-mediated induction of CYP3A4. In fact, dexamethasone has been shown to be a true ligand of the human PXR but only at supramicromolar concentrations (>10 μM) [15]. This is consistent with our observation that the CYP3A4 mRNA increase in response to high (supramicromolar) concentrations of dexamethasone displays similar kinetics and amplitude to those observed with micromolar concentrations of rifampicin. Such high

Table 1
Shared xenobiotic modulators of the human receptors involved in CYP2B6, CYP2C9, and CYP3A4 gene regulation

Compounds	GR	PXR	CAR	CYPs induced in PCHH
Dexamethasone, nM range	+			CYP3A4, CYP2C8/9
Dexamethasone, μM range	+	+		CYP3A4
PCN	–			none
RU486	–	+		CYP3A4
Androstenol		+	–	CYP2B6, CYP3A4
Progesterone		+	–	?
Clotimazole		+	–	CYP3A4, CYP2C8/9
5β-Pregnane 3,20-dione		+	+	?
Estrogens		+	+	?

+: activator.
– : repressor.
PCHH: primary cultures of human hepatocytes.

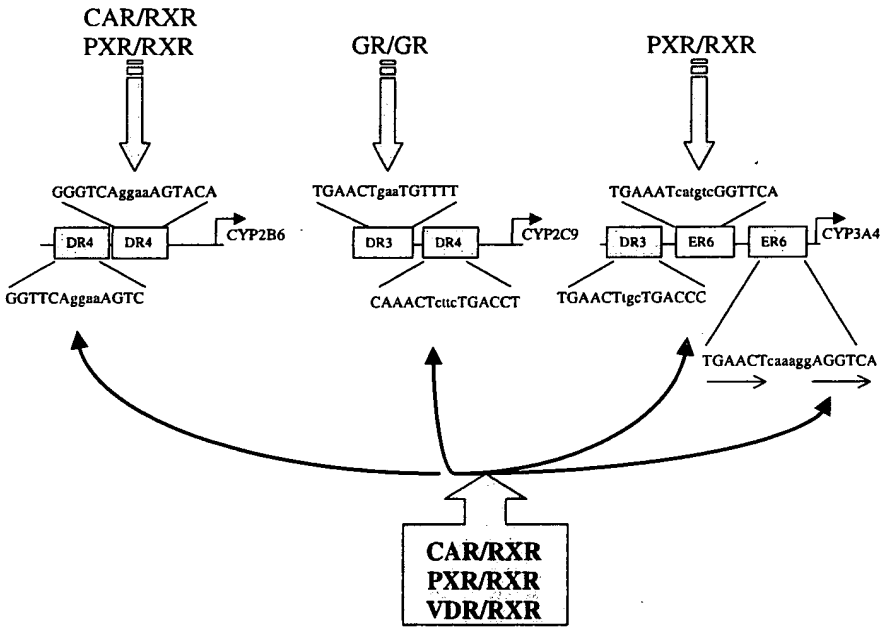

Fig. 1. Shared response elements of the receptors involved in CYP2B6, CYP2C9 and CYP3A4 gene regulation.

concentrations of glucocorticoids are not likely to be relevant under normal physiological conditions. However, they might be reached either under bolus administration of glucocorticoids (in graft rejection episodes) or in stress situations. High concentrations of glucocorticoids have been shown to cause tissue injury [54–56]. As these compounds are primarily metabolized by CYP3A4 to yield inactive and readily excretable metabolites such as 6-hydroxy-derivatives, it is possible that this high-glucocorticoid component of CYP3A4 induction represents a physiological autoregulatory process aimed at eliminating these hormones efficiently, and thus preventing further tissue and cellular injury.

Crystal structure analyses suggest that the ligand binding domain of the human PXR is highly hydrophobic and flexible, allowing molecules of different size to bind in multiple orientations [57]. Another interesting feature of PXR is the species-specificity of the ligand activators which correlates with the species-specificity displayed by CYP3A induction in response to these activators [58].

Studies in PXR-null mice suggest that PXR is part of a protective mechanism against secondary bile acid-induced liver injury, in part through its role as an inducer of hepatic CYP3A4 gene expression [59–61]. When mice are pretreated with the rodent PXR activator PCN, they are protected against lithocholic acid-induced liver injury. In contrast, this protective effect of PCN is absent in PXR-null mice. Screening of bile acids identified lithocholic acid and 6-keto lithocholic acid as PXR ligands [62]. In addition, it has been observed in PXR-null mice experiments that PXR is a negative regulator of the cyp7a1 gene [60], the rate-limiting step in bile acid biosynthesis. According to these data, PXR has been proposed as a secondary bile acid receptor, involved in the regulation of cholesterol catabolism and bile acid homeostasis.

3. The CAR

The CAR is an orphan receptor, which was originally characterized as a constitutive activator of retinoid acid response elements (RARE). It is called ≪constitutive≫ because of its ability to transactivate RAREs and other response elements in the absence of ligand [63]. CAR is predominantly expressed in the liver and intestine [63] and it mediates the phenobarbital induction of UDP-glucuronosyltransferase (UGT1A1) [64], CYP2B6 [50], CYP3A4 [7,50] and CYP2C9 [31] through DR4 motifs (Fig. 1), and enoyl-CoA hydratase/3-hydroxy acyl CoA-dehydrogenase gene through a DR1 motif [65]. In mice, increased liver weight and DNA synthesis in response to phenobarbital treatment were also shown to be mediated by CAR [66]. CAR transactivates the PXR-responsive ER6 and DR3 motifs in the CYP3A4 gene [7,50]. This is consistent with the finding that phenobarbital can induce CYP3A4 mRNA in PXR-null mice [67]. Targeted in vivo disruption of CAR in mice completely abrogates phenobarbital- and 1,4-bis (2-(3,5-dichloropyridyloxy))benzene (TCPOBOP)-mediated induction of cyp2b10 and cyp3a genes, demonstrating the pivotal role of this receptor in the xenobiotic induction of some P450s.

Phenobarbital has long been known to induce drug metabolism in laboratory animals and man. Many other structurally diverse drugs and chemicals display similar profiles of enzyme induction [68,69]. The phenobarbital responsive element module (PBREM) has been characterized initially in the far upstream region of rat CYP2B [70]. The PBREM usually consists of direct repeats of the nuclear receptor binding site. The identification of CAR as the mediator of phenobarbital action on CYPs expression came from two independent experiments: (1) transient transfection of various nuclear receptors in cell lines showed that only CAR was able to activate PBREM-dependent reporter gene [71], and (2) both protein binding assay and DNA affinity purification from mouse liver nuclear extracts showed that CAR and RXR could bind to a DR4 element immobilized on a column only after in vivo phenobarbital treatment [71]. HepG2 or HuH7 cell lines that were genetically modified in such a way as to constitutively express CAR were shown to re-express CYP2B6 [50] and CYP3A4 [15].

CAR acts differently from the more traditional receptors: a key step in its transcriptional activation is its nuclear translocation. In mice, in vivo, CAR accumulates in the hepatocyte nucleus only after phenobarbital treatment [24,72]. We made a similar observation in cultured human hepatocytes [20, and Pascussi, unpublished data]. The molecular mechanisms involved in CAR nuclear translocation are still unclear, but seem to involve a specific phosphorylation-sensitive event [72,73]. The phenobarbital-induced CAR nuclear translocation is uncoupled by concomitant exposure to okadeic acid, an inhibitor of phosphatase 1 and 2A [72], suggesting that dephosphorylation of mCAR is required for this process. Indeed, previous reports have documented the importance of phosphorylation status in CYP2B induction [74,75]. In addition, nuclear translocation of CAR seems to be dependent on a leucine-rich region near the C terminus of the protein that has been designated a xenochemical response signal [76].

CAR has been shown to mediate gene induction in response to phenobarbital and phenobarbital-like inducers in rodents and man. However, in man, the only activator shown to bind hCAR is 5β-pregnane, while phenobarbital is not a ligand in vitro [77]. Deactivators or inverse agonists, such as androstenol [78] or clotrimazole [77], also bind hCAR. Indeed, many PXR activators are also CAR activators (phenobarbital, 5β-pregnane) or deactivators (clotrimazole, androstenol, progesterone), and this constitutes an unresolved part of the properties of these xenosensors [77] (Table 1). In contrast to PXR, CAR is not activated by bile acids, but some of these including cholic acid, 6-ketolithocholic acid and 7-ketodeoxycholic acid methyl ester act as transrepressors of CAR [62].

Recently, progesterone (a PXR activator) and androgens have been shown to inhibit the transcriptional activity of CAR, while active estrogens (estradiol and estrone) are effective activators [79]. The regulation of CAR by androgens, estrogens and progesterone is somewhat intriguing and remains to be clarified. However, these observations bring new insight into the roles of this receptor in the endocrine modulation of phenobarbital induction in the liver in vivo. Indeed, in WKY rats, phenobarbital induces CYP2B genes in males while females respond only poorly [80,81].

4. The VDR

The biologically active form of vitamin D3, 1α,25-dihydroxy vitamin D3, is an important regulator of cell growth, differentiation and death. The cellular action of 1α,25-dihydroxy vitamin D3 is known to be mediated via an intracellular receptor, the VDR, a member of the superfamily of steroid receptors (VDR, NR1I1). Ligand-activated VDR provokes partial arrest in G_0/G_1 of the cell cycle, induction of differentiation and control of calcium homeostasis [82]. Although the liver is the site of the 25-hydroxylation of vitamin D, it has been shown to have a very low proportion of VDRs and, consequently, was not considered as a target site of vitamin D action. However, studies have demonstrated that calcium and/or vitamin D deficiency has a significant effect on liver cell physiology [83].

The implication of the VDR in CYP3A4 gene expression was first suggested by Schmiedlin-Ren et al. [22]. These authors showed that 25-hydroxy vitamin D3 behaves as a transcriptional inducer of CYP3A4 in the colic carcinoma cell line Caco-2. Moreover, the extent of CYP3A4 induction by vitamin D3 derivatives correlated with their affinity for binding to VDR, with the following rank order: 1α,25-dihydroxy vitamin D3>25-hydroxy vitamin D3>unhydroxylated vitamin D3 [84]. Finally, ligand-activated VDR has been shown to induce the expression of CYP2B6, CYP2C9 and CYP3A4 mRNAs in human hepatocytes [21], while others have reported that 1α,25-dihydroxy vitamin D3 controls the transcription of CYP3A4 in the human intestinal LS180 cell line [23]. PXR is closely related to VDR since these receptors share 63% homology of amino acid sequence in their DNA binding domains. However, their ligand binding domains exhibit only 37% identity. The role of PXR in mediating vitamin D3 effects seems to be excluded as: (i) hPXR is not activated by vitamin D and its hydroxy derivatives; and (ii) hPXR expression is not increased by these compounds.

Vitamin D3 function is mediated through VDR which forms a heterodimer with RXR after ligand binding [85]. This heterodimer then binds to and transactivates vitamin D response elements (VDRE) present in the regulatory region of target genes. The classical VDRE consists of an imperfect direct repeat of a core hexanucleotide sequence (G/A)GGT(G/C)A, with three-nucleotide spacers. CYP2B6, CYP2C9 and CYP3A4 gene promoters lack such a motif according to published sequences. However, the heterodimer VDR/RXRα binds the PXR/CAR responsive elements located upstream of the CYP2B6, CYP2C9 and CYP3A4 genes [22] (Fig. 1). Finally, the promiscuity of these receptors in the induction of CYPs by xenobiotics and endogenous compounds is expected to generate many interferences between their respective signalling pathways. Indeed, we observed that VDR-mediated CYP3A4 reporter gene induction was inhibited by PXR or CAR co-transfection [21].

The reason why expression of CYP genes is controlled by VDR is unclear. CYP2B6, CYP2Cs or CYP3A4 have not been shown to be involved in the metabolism of vitamin D. The major route of degradation of vitamin D is the oxidation of the side chain of the molecule, catalyzed by vitamin D-24 hydroxylase (CYP24), an enzyme which is highly induced by 1α,25 dihydroxy vitamin D and its other derivatives [86]. However, prolonged therapy with rifampicin causes vitamin D deficiency [87–90]. In eight healthy subjects, rifampicin treatment reduced circulating levels of 25-hydroxy vitamin D and 1α,25-dihydroxy vitamin D by 34% and 23%, respectively. In addition, rifampicin and phenobarbital are two of the drugs most frequently associated with osteomalacia, a metabolic bone disease characterized by a defect in bone mineralization frequently due to an alteration of vitamin D metabolism. This suggests that CAR and/or PXR might be involved in the control of the genes responsible for the synthesis or catabolism of vitamin D.

5. The GR

A controversial and not yet fully resolved issue has been the role of the GR on the mechanism of CYP induction by glucocorticoids. Based on GR-deficient mice, Schuetz et al. [91] proposed that the GR is not required for the induction of CYP3A by glucocorticoids in this species while its expression is essential for the induction of CYP2B. However, extrapolating data from rodents to man is hazardous because of the species-specificity in the response of CYPs and nuclear receptors to xenobiotics [58,92]. Several lines of evidence suggest that CAR, PXR and VDR may not totally account for steroid induction of CYP2s and CYP3A, and the possibility exists that the GR is involved in some way in the induction of these genes. The following points are of note in this respect: (i) induction of endogenous human CYP2B6, CYP2C8/9 and CYP3A4 in cultured hepatocytes [20,93] is potentiated by pre-treatment of cells with dexamethasone; (ii) the response to glucocorticoids of a CYP3A4 promoter-dependent gene reporter is increased in the presence of cotransfected hGR [52]; (iii) transcriptional activation by dexamethasone of rat CYP3A1 and human CYP3A4 promoters [15,32,38] is blocked by the antiglucocorticoid mifepristone (RU486), a mouse and human PXR

activator [27,28]; (iv) induction of CYP3A4 and CYP2C9 mRNA expression by dexamethasone in cultured human hepatocytes is inhibited by submicromolar concentrations of RU486 while this compound induces CYP3A4 mRNA only at micromolar concentrations, suggesting the involvement of at least two distinct pathways in the response of this gene to agonist and antagonist glucocorticoids [15,31].

On the other hand, several other lines of evidence plead in favor of the role of GR in CYP gene regulation. For instance, the human CYP3A5 gene promoter contains two glucocorticoid response elements (GRE), separated by 160 bp, which confer glucocorticoid response to reporter genes in HepG2 cell [94]. In contrast to CYP3A4 and CYP3A7, the CYP3A5 promoter has no functional PXR binding site in its proximal region [26] but is still inducible by glucocorticoids [95]. Moreover, a functional GRE in the CYP2C9 gene promoter has recently been reported to trigger the GR-mediated CYP2C9 gene induction by glucocorticoids, while PXR failed to transactivate this homologous construct [31]. Furthermore, it had previously been reported that GR binds to a GRE present in the rat CYP3A1 gene, and it has been suggested that cooperation of the upstream GRE and downstream elements (e.g. PXR element) may be required for the maximal response of CYP3A to glucocorticoids [96].

In human hepatocyte cultures, the GR appears to be constitutively expressed and its level is not affected by glucocorticoids and other xenobiotics. We recently reported that in these cultures glucocorticoids (dexamethasone, hydrocortisone, prednisolone) increase the levels of CAR, PXR and RXRα mRNAs and proteins, leading to the potentiation of xenobiotic-mediated induction of CYP2B6, CYP2C8/9 and CYP3A4 [20,93]. This effect is likely to be mediated through the direct transcriptional activation of these genes by GR. The following points are of note in this respect: (i) dexamethasone does not affect the rate of degradation of the nuclear receptor mRNAs; (ii) induction of PXR and CAR mRNAs is partially blocked by the glucocorticoid antagonist RU-486; and (iii) cycloheximide does not affect the nuclear receptor expression. Moreover, in these cultures, dexamethasone produces a biphasic induction of CYP3A4 mRNA consisting of a low-dexamethasone component (nanomolar concentrations) of low amplitude, followed by a high-dexamethasone component (supramicromolar concentrations) of high amplitude. We proposed that while PXR is involved in the supramicromolar range of dexamethasone-induced CYP3A4 induction (see PXR section), the GR indirectly controls the major part of the low-dexamethasone component of CYP3A4 mRNA induction by increasing the expression of PXR, CAR and RXR. Thus, at nanomolar concentrations of dexamethasone, CYP3A4 mRNA induction is a secondary GR-mediated response. The following observations support this hypothesis: (i) cotransfection of either PXR or CAR in HepG2 cells, in the absence of any exogenous ligand, significantly activates CYP3A4 promoter reporter gene expression; (ii) in HepG2 cells co-transfected with GR, CYP3A4 promoter activity is increased only after 48 h of treatment with submicromolar concentrations of dexamethasone, that is, in parallel with the expression of both CAR and PXR mRNAs; (iii) CYP3A4 mRNA becomes detectable in HepG2 cells transfected by CAR, in the absence of any exogenous ligand; (iv) in primary cultures of human hepatocytes, nanomolar concentrations of dexamethasone trigger the nuclear translocation of CAR and increased accumulation of PXR in the absence of any exogenous xenobiotic.

These studies lead to the elaboration of a comprehensive model for the involvement of GR which is consistent with the pharmacological characteristics of the response of CYP genes to glucocorticoids (Fig. 2). According to this model, the response to glucocorticoids may be divided into two steps involving two receptors depending on the glucocorticoid concentration: (i) GR for physiological doses (submicromolar) and (ii) PXR for stress-induced or pharmacological (bolus) doses (micromolar or supramicromolar). Physiological levels of glucocorticoids activate GR and increase the production of PXR, CAR and RXR, which in turn can further function as rate-limiting positive transcription regulators for glucocorticoid- and xenobiotic-mediated responses. This is consistent with our observation that in a bank of human liver tissue, CYP3A4 mRNA levels correlated significantly with both PXR and CAR levels (Pascussi, unpublished data). This suggests that these receptors might be functional even in the absence of added ligand, probably due to the presence of endogenous activator compounds. In favor of this is the observation [29,30] that the basal expression of CYP3A is drastically reduced in transgenic mice deficient in RXRα, the necessary partner of both CAR and PXR for DNA binding and transcriptional activity. Moreover, the circadian variation of the hepatic CYP3A4 activity reported in man [97] mirrors cortisol in its circadian fluctuation. In human serum, the normal level of hydrocortisone (the major circulating glucocorticoid) ranges from 0.1 to 0.45 μM, a concentration that permits the full activation of GR. This suggests therefore that hydrocortisone may be responsible for the expression of RXR, CAR and PXR, thus mediating indirectly the basal expression of CYP3A4.

There are conflicting data in the literature regarding the contribution of glucocorticoids to the phenobarbital induction of CYP2B. Although most reports indicate that phenobarbital induction of rat or mouse CYP2B either requires or is potentiated by glucocorticoids [79,94,100], high concentrations of dexamethasone can suppress phenobarbital induction of CYP2B in rats, most likely because of toxic effects [37]. There is also evidence that GR may participate in the regulation of CYP2B genes. For example, mouse CYP2B10 and rat CYP2B1/2 genes contain GREs [98], and rat CYP2B2 GRE is activated by glucocorticoids [99]. Moreover, RU486 inhibits phenobarbital induction of rat CYP2B [100] and chicken CYP2H1 [101] genes. There is an indication as well that the GR is required for maximum induction by phenobarbital of CYP2B10 in mice and of

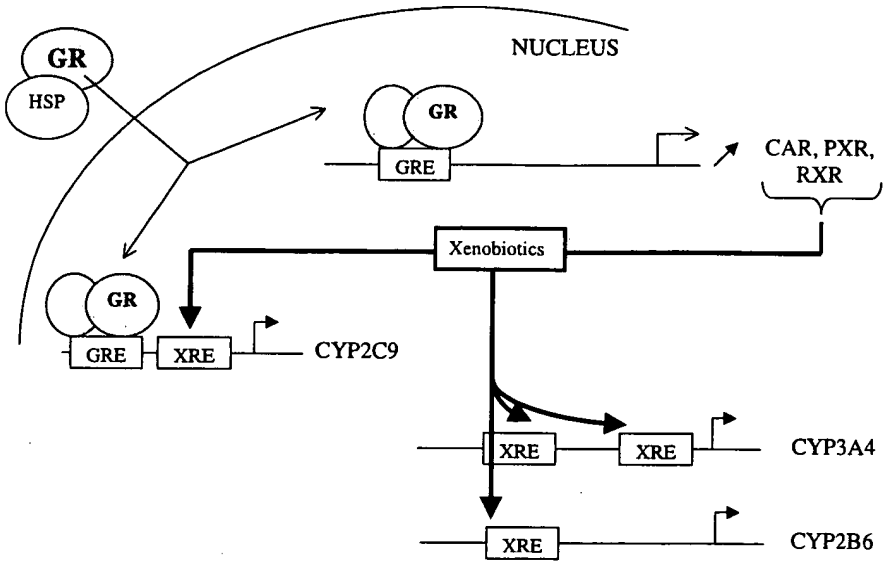

Fig. 2. Model summarizing the proposed roles of GR in the transcriptional activation of CYP2B6, CYP2C9 and CYP3A4. The pathway can be divided into two steps. First, physiological concentrations of glucocorticoids increase cellular CYP2C9 (and CYP3A5) but also CAR, RXR and PXR levels via a GR-mediated mechanism. This leads to an increased expression of rate-limiting transcription factors possibly responsible for the low level of basal transcriptional activation of CYP3A4. Second, PXR and CAR are activated by xenochemicals or high concentrations of glucocorticoids (PXR) resulting in an enhancement of CYP2B6, CYP2C9 and CYP3A4 gene expression. GRE: glucocorticoid response element; XRE: xenobiotic response element.

CYP2B2 in rats [74]. Recently, using mice with a targeted mutation in the GR, Schuetz and al. [91] reported that the GR is essential for phenobarbital-mediated induction and basal expression of the CYP2B10 gene in mice. Moreover, glucocorticoids are necessary for CYP2B6, CYP2C8, CYP2C9 and CYP3A4 induction by phenobarbital in human hepatocytes [20]. A putative GRE has been identified as part of a phenobarbital response unit in the 5′-flanking sequence of the rat CYP2B2 gene [98]. However, this motif is not present in the CYP2B6, CYP2C9 and CYP3A4 xenobiotic-responsive elements. In addition, CYP2B6 is not induced by glucocorticoids in cultured human hepatocytes, while glucocorticoids potentiate its induction by phenobarbital. We recently elucidated the molecular mechanism of this synergy. As for the PXR, we found that the ligand-activated GR controls the expression of CAR [20]. Therefore, physiological levels of glucocorticoid activate the GR and this leads to an increase the production of the CAR protein. Cloning of the CAR promoter and identification of functional GREs are currently under investigation, and preliminary results support this theory (Pascussi, personal unpublished data).

6. Discussion

Several possibilities can be proposed to explain how a single CYP might be induced by different classes of chemicals: (i) many structurally unrelated chemicals can bind to the same receptor, as observed with PXR, or (ii) CYP genes might contain multiple xenobiotic response elements, each of which binds a distinct nuclear receptor. However, actual data suggest that distinct receptors may share the same response elements as observed with CAR, PXR and VDR (Fig. 1). Indeed, recent studies have demonstrated that PXR and CAR have the potential to cross-regulate CYP gene expression by two independent mechanisms: (i) binding of PXR and CAR to each other's DNA response element, and (ii) sharing some ligands and/or activators. This is consistent with recent studies in PXR- and CAR-knockout mice emphasising the overlapping role of these receptors on the induction mechanism of detoxifying enzymes and transporters. Interestingly, it has been suggested that CAR and PXR evolved from the same ancestral gene CXR (Chicken Xenobiotic-sensing Receptor) and have then diverged during the species evolution from birds to man with the conservation of signalling pathways mediated by these xenobiotic-sensing orphan receptors [62,102].

The most intriguing question is how a single chemical can differentially induce more than one CYP. For example, rat CYP3A1 exhibits a threefold increased expression in response to phenobarbital while, in the meantime, CYP2B1 exhibits a 50- to 100-fold increase [103]. Similarly, CYP2B1 exhibits a threefold increased expression in response to PCN, a rodent PXR ligand, while in the meantime CYP3A1 exhibits a 10-fold increase [103]. Moreover, the time course of induction of CYP2B and CYP3A differs after treatment with PCN or phenobarbital [104] and induction of these cytochromes can be differentiated by the dose of the inducer [105]. This differential induction of CYP genes by the same xenobiotic may be the result of the use of different receptors. Indeed, as mentioned in previous sections, these receptors

share activators, i.e. dexamethasone (GR/PXR), phenobarbital and 5β-pregnane (CAR/PXR). In addition, an activator for one receptor might be a deactivator for another receptor, i.e. RU486 (GR/PXR) and clotrimazole/androstenol (CAR/PXR) (Table 1). The binding of these chemicals is likely to induce different receptor conformations leading to different transcriptional potency depending on the receptor. In addition, these receptors might bind the same response element with different affinities. For example, the CAR/RXR heterodimer binds the rat CYP2B1 DR4 more efficiently than does the PXR/RXR, and the PXR/RXR binds more efficiently the rat CYP3A1 proximal DR3 PXRE than does the CAR/RXR [8]. Therefore, the nature of the DNA response element may influence both the binding affinity of the receptor heterodimer and its ability to interact with the transcriptional machinery, and thus may provide a basis for the differential induction. However, studies comparing CAR/RXR with PXR/RXR binding to each other's response element established that human CAR and PXR exhibit similar binding affinity towards the response elements controlling the CYP3A4 gene.

In addition, differences in CYP induction response may be due, in part, to the relative abundance of these receptors, as they compete for cofactors (such as SCR-1, and RXRα) and ligands. The extent of such cross-talk would be gene- and inducer-specific and would depend on parameters such as the binding affinity of the receptor to specific DNA elements and ligands, and on the expression levels of these receptors in the cell. The existence of multiple xenobiotic receptors with distinct but overlapping ligand specificity increases the organism's ability to detect and respond to a potentially harmful substance. Moreover, endogenous substances or xenobiotics might alter the absolute (or relative) amount of a given receptor. This can result from an alteration of either the receptor expression or its rate of degradation. As noted in the previous section, glucocorticoids induce CAR, RXR and PXR expression, leading to an enhancement of their biological action. However, it has recently been demonstrated that certain members of the nuclear hormone receptor superfamily are degraded through the ubiquitin–proteasome pathway in a ligand-dependent manner. Notably, Masuyama et al. [106] recently showed that PXR interacts with SUG1, a component of the proteasome, in a progesterone-dependent manner, and the use of proteasome inhibitors confirmed that PXR degradation proceeds through this pathway. However, they observed that endocrine-disrupting chemicals such as phthalic acid and nonylphenol, which activate PXR-mediated transcription, do not enhance this interaction, but instead slow the rate of proteasome-mediated degradation of PXR, in contrast to what is observed in the presence of progesterone. On the other hand, 1α,25-dihydroxy vitamin D3 increases the level of the nuclear vitamin D3 receptor by blocking the ubiquitin/proteasome-mediated degradation of this receptor in man [107]. This suggests that endogenous or exogenous compounds may affect the level of receptor proteins, leading to either increased or decreased accumulation, in a specific manner depending on the receptor and the compound.

In conclusion, the adaptive system of protection against xenochemicals appears now as a tangle of networks, where receptors share partners, ligands, DNA response elements and target genes. In addition, the net effect of a xenobiotic on gene transcription will often be complex and depend on its effects on both the activation and expression of these receptors.

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EXHIBIT 3

Selective activation of vitamin D receptor by lithocholic acid acetate, a bile acid derivative[S]

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Abstract **The vitamin D receptor (VDR), a member of the nuclear receptor superfamily, mediates the biological actions of the active form of vitamin D, 1α,25-dihydroxyvitamin D_3. It regulates calcium homeostasis, immunity, cellular differentiation, and other physiological processes. Recently, VDR was found to respond to bile acids as well as other nuclear receptors, farnesoid X receptor (FXR) and pregnane X receptor (PXR). The toxic bile acid lithocholic acid (LCA) induces its metabolism through VDR interaction. To elucidate the structure-function relationship between VDR and bile acids, we examined the effect of several LCA derivatives on VDR activation and identified compounds with more potent activity than LCA. LCA acetate is the most potent of these VDR agonists. It binds directly to VDR and activates the receptor with 30 times the potency of LCA and has no or minimal activity on FXR and PXR. LCA acetate effectively induced the expression of VDR target genes in intestinal cells. Unlike LCA, LCA acetate inhibited the proliferation of human monoblastic leukemia cells and induced their monocytic differentiation. We propose a docking model for LCA acetate binding to VDR. The development of VDR agonists derived from bile acids should be useful to elucidate ligand-selective VDR functions.**—Adachi, R., Y. Honma, H. Masuno, K. Kawana, I. Shimomura, S. Yamada, and M. Makishima. **Selective activation of vitamin D receptor by lithocholic acid acetate, a bile acid derivative.** *J. Lipid Res.* **2005.** 46: **46–57.**

Supplementary key words nuclear receptor • structure-function relationship • colon cancer • intestine • leukemia

The active form of vitamin D, 1α,25-dihydroxyvitamin D_3 [1,25(OH)2D3], regulates calcium homeostasis, immunity, and cellular growth and differentiation (1). 1,25(OH)2D3 has been demonstrated to be able to inhibit the proliferation and/or to induce the differentiation of various types of malignant cells, including breast, prostate, colon, skin, and brain cancer cells, as well as myeloid leukemia cells in vitro (2). The administration of 1,25(OH)2D3 or its analogs has therapeutic effects in mouse models of malignancies such as leukemia and colon cancer (3, 4). The biological activities of 1,25(OH)2D3 are mediated through binding to its intracellular receptor, the vitamin D receptor (VDR; NR1I1), a member of the nuclear receptor superfamily. Additional nongenomic mechanisms of action may be mediated by a poorly characterized membrane receptor (5). Structure-function relationship studies of the interaction of vitamin D analogs with VDR reveal different binding modes in the ligand-binding pocket of VDR (6). These differences in ligand-receptor interaction may contribute to the differential recruitment of coactivators to VDR and selective biological actions (7).

Nuclear receptors are ligand-inducible transcription factors that are involved in many biological processes, including cell growth and differentiation, embryonic development, and metabolic homeostasis (8). Recently, nuclear receptors belonging to the NR1H and NR1I subfamilies, including VDR, have been shown to control cholesterol

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Abbreviations: BSEP, bile salt export pump; CAR, constitutive androstane receptor; ER, estrogen receptor; FXR, farnesoid X receptor; IBABP, ileal bile acid binding protein; 3-keto-LCA, 3-keto-cholanic acid; LCA, lithocholic acid; LXR, liver X receptor; NBT, nitroblue tetrazolium; N-CoR, nuclear receptor corepressor; 1,25(OH)2D3, 1α,25-dihydroxyvitamin D_3; PPAR, peroxisome proliferator-activated receptor; PXR, pregnane X receptor; RAR, retinoic acid receptor; RXR, retinoid X receptor; SRC-1, steroid receptor coativator-1; TR, thyroid hormone receptor; VDR, vitamin D receptor.

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and bile acid metabolism (9). Liver X receptor α (LXRα; NR1H3) and LXRβ (NR1H2) function as oxysterol receptors and regulate cholesterol metabolism in liver, intestine, adipose tissue, and macrophages. Bile acids, which are major metabolites of cholesterol in the body, bind to farnesoid X receptor (FXR; NR1H4) and induce negative feedback regulation of their synthesis from cholesterol. Primary bile acids, produced in the liver, are excreted in the bile after conjugation with taurine and glycine and are subsequently reabsorbed in the intestine. Bile acids that escape reabsorption are converted to secondary bile acids by the intestinal microflora. Pregnane X receptor (PXR; NR1I2), which acts as a receptor for various xenobiotics, senses the levels of secondary bile acids and induces their metabolism in the liver (10, 11). VDR was also found to function as a receptor for secondary bile acids such as lithocholic acid (LCA) and to be involved in bile acid metabolism by inducing a LCA detoxification mechanism in the liver and intestine (12).

Previously, we analyzed the structure-function relationships of the endocrine [1,25(OH)2D3] and xenobiotic (LCA) ligands with VDR and revealed that 1,25(OH)2D3 and LCA interact with a different set of amino acids in the ligand binding pocket of VDR (13, 14). These results suggest the possibility that VDR adopts distinct conformations in response to 1,25(OH)2D3 and LCA binding and provides a possible mechanism for the compounds' different biological actions. The docking models of LCA and 3-keto-cholanic acid (3-keto-LCA), which is a metabolite of LCA, reveal that these compounds are accommodated in the VDR ligand binding pocket more weakly than 1,25(OH)2D3 (13, 14), suggesting that modification of these bile acids can increase the VDR transactivation activity. In this study, we examined the ability of several LCA analogs to activate VDR and found that modification of the 3 position of LCA increased VDR transactivation by more than 30-fold. Furthermore, the LCA acetate analog can induce differentiation of myeloid leukemia cells.

MATERIALS AND METHODS

Chemical compounds

LCA formate and LCA isobutyrate were synthesized in our laboratory (H. Masuno and S. Yamada, unpublished results), and other bile acids and derivatives were purchased from Sigma-Aldrich (St. Louis, MO), Wako (Osaka, Japan), Nacalai (Kyoto, Japan), or Steraloids (Newport, RI). 1,25(OH)2D3 was obtained from Calbiochem (San Diego, CA).

Plasmids

Fragments of human VDR (GenBank accession number NM_000376), FXR (accession number NM_005123), and PXR (accession number NM_022002) were inserted into the pCMX vector to make pCMX-VDR, pCMX-FXR, and pCMX-PXR, respectively (12, 14, 15). The ligand binding domains of human VDR, FXR, thyroid hormone α1 (TRα1) (accession number NM_199334), retinoic acid receptor α (RARα) (accession number NM_000964), LXRα (accession number NM_005693), constitutive androstane receptor (CAR) (accession number NM_005122), estrogen receptor α (ERα) (accession number NM_000125), retinoid X receptor (RXRα; accession number NM_002957), and mouse peroxisome proliferator-activated receptor α (PPARα; accession number NM_011144), PPARδ (accession number NM_011145), and PPARγ (accession number NM_011146) were inserted into the pCMX-GAL4 vector to make pCMX-GAL4-VDR, pCMX-GAL4-FXR, pCMX-GAL4-TRα, pCMX-GAL4-RARα, pCMX-GAL4-LXRα, pCMX-GAL4-CAR, pCMX-GAL4-ERα, pCMX-GAL4-RXRα, pCMX-GAL4-PPARα, pCMX-GAL4-PPARδ, and pCMX-GAL4-PPARγ, respectively. A full-length fragment of human VDR was inserted into the pCMX-VP16 vector to make pCMX-VP16-VDR. Nuclear hormone receptor-interacting domains of steroid receptor coactivator-1 (SRC-1) (amino acids 595–771; GenBank accession number U90661) and nuclear receptor corepressor (N-CoR) were inserted into the pCMX-GAL4 vector to make pCMX-GAL4-SRC-1 and pCMX-GAL4-N-CoR (14). Mutations were introduced into pCMX-GAL4-VDR using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). hCYP3A4-ER-6x3-tk-LUC, IR-1x3-tk-LUC, and GAL4-responsive MH100 (UAS)x4-tk-LUC reporters were used to evaluate the activities of VDR and PXR, FXR, and GAL4-chimera receptors, respectively. All plasmids were sequenced before use to verify DNA sequence fidelity.

Cell lines and cell culture

HEK293 cells were cultured in Dulbecco's modified Eagle's medium containing 5% fetal bovine serum and antibiotic-antimycotic (Nacalai) at 37°C in a humidified atmosphere of 5% CO_2 in air. Human hepatoblastoma HepG2 cells and colon cancer SW480 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and antibiotic-antimycotic (Nacalai) at 37°C in a humidified atmosphere of 5% CO_2 in air. Human myeloid leukemia THP-1 cells were cultured in suspension in RPMI 1640 medium containing 10% fetal bovine serum and 80 μg/ml gentamicin at 37°C in a humidified atmosphere of 5% CO_2 in air (16).

Cotransfection assay

Transfections were performed by the calcium phosphate coprecipitation assay as described previously (14). Eight hours after transfection, test compounds were added. Cells were harvested 16–24 h later, and luciferase and β-galactosidase activities were assayed using a luminometer and a microplate reader (Molecular Devices, Sunnyvale, CA). Cotransfection experiments used 50 ng of reporter plasmid, 20 ng of pCMX-β-galactosidase, 15 ng of each receptor and/or cofactor expression plasmid, and pGEM carrier DNA to give a total of 150 ng of DNA per well of a 96-well plate. Luciferase data were normalized to the internal β-galactosidase control and represent means ± SD of triplicate assays.

Competitive ligand binding assay

Human VDR protein was generated using the TNT Quick Coupled Transcription/Translation System (Promega, Madison, WI). The protein was diluted 5-fold with ice-cold TEGWD buffer (20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol, 20 mM sodium tungstate, and 10% glycerol). The diluted lysate was incubated with 1 nM of [26,27-methyl-^{3}H]1,25(OH)2D3 for 16 h at 4°C in the presence or absence of nonradioactive competing compounds (14). Bound and free compounds were separated by the dextran-charcoal method (17). Bound and labeled 1,25(OH)2D3 was quantitated using scintillation counting.

Graphic manipulation and docking

Graphic manipulations were performed using SYBYL 6.8 (Tripos, St. Louis, MO) (13, 14). The atomic coordinates of the human VDR ligand binding domain (Δ165–215) crystal structure were retrieved from the Protein Data Bank (PDB #1DB1). LCA

acetate was docked into VDR using the docking software FlexX (version 1.10; Tripos) (18).

Animal studies

C57BL/6J mice were obtained from Japan SLC (Hamamatsu, Japan) and were maintained under controlled temperature (23 ± 1°C) and humidity (45–65%) with free access to water and chow (Oriental Yeast, Tokyo, Japan). Experiments were conducted with male mice between 8 and 9 weeks of age. Mice were treated orally with LCA or LCA acetate in a polyethylene glycol-Tween 80 (4:1) formulation or with vehicle alone (19). Mice were analyzed 12 h after treatment under fasting conditions. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Osaka University.

Quantitative real-time RT-PCR analysis

Total RNAs from samples were prepared with an RNA STAT-60 kit (Tel-Test, Friendswood, TX). The cDNA was synthesized using the ThermoScript RT-PCR System (Invitrogen, Carlsbad, CA). Real-time PCR was performed on a LightCycler using the Fast-Start DNA Master SYBR Green I (Roche Diagnostics, Tokyo, Japan) according to the instructions provided by the manufacturer (19). Primers were as follows: human VDR, 5′-GCTGACCTGGTCAGTTACAGCA-3′ and 5′-CACGTCACTGACGCGGTACTT-3′; RXRα, 5′-AAGATGCGGGACATGCAGAT-3′ and 5′-CAGGCGGAGCAAGAGCTTAG-3′; cyclophilin, 5′-CCCACCGTGTTCTTCGACAT-3′ and 5′-CCAGTGCTCAGAGCACGAAA-3′; CYP24A1, 5′-TGAACGTTGGCTTCAGGAGAA-3′ and 5′-AGGGTGCCTGAGTGTAGCATCT-3′; CYP3A4, 5′-AGTGTGGGGCTTTTATGATG-3′ and 5′-ATACTGGGCAATGATAGGGA-3′; CaT1, 5′-AGCCTACATGACCCCTAAGGACG-3′ and 5′-GTAGAAGTGGCCTAGCTCCTCGG-3′; E-cadherin, 5′-GAAGGTGACAGAGCCTCTGGATAG-3′ and 5′-CTGGAAGAGCACCTTCCATGA-3′; bile salt export pump (BSEP), 5′-TCTTTACTGGATTCGTGTGG-3′ and 5′-TGACACTGAGGAAAATCTGG-3′; mouse cyclophilin, 5′-CAGACGCCACTGTCGCTTT-3′ and 5′-TGTCTTTGGAACTTTGTCTGCAA-3′; Cyp24a1, 5′-CCCATTACTCAGGGAAGCAC-3′ and 5′-CCACTCAGACAATGAAGCCA-3′; Cyp3a11, 5′-CCAACAAGGCACCTCCCACG-3′ and 5′-TGGAATTCTTCAGGCTCTGA-3′; ileal bile acid binding protein (IBABP), 5′-GGTACCACCATGGCCTTCAGTGGCAAATAT-3′ and 5′-GCTAGCTCAAGCCAGCCTCTTGCTTAC-3′. The RNA values were normalized to the amount of cyclophilin mRNA and are represented in arbitrary units.

Growth and differentiation of myeloid leukemia cells

Cell suspensions were cultured with or without test compound. The cells were counted in a model ZM Coulter Counter (Coulter Electronics, Luton, UK). Cell morphology was examined in cell smears stained with May-Gruenwald-Giemsa. α-Naphthyl acetate esterase was determined cytochemically, nitroblue tetrazolium (NBT) reduction was assayed colorimetrically, and expression of monocytic antigens CD11b and CD14 on the cell surface was determined using indirect immunofluorescent staining and a flow cytometer (Epics XL; Coulter Electronics) (16, 20).

RESULTS

Transactivation of VDR by LCA derivatives

To elucidate the structure-activity relationship of LCA derivatives (**Fig. 1A**) on VDR function, we transiently transfected HEK293 cells with a VDR expression vector and a luciferase reporter containing a VDR-responsive everted repeat-6 element from the CYP3A4 promoter (12). Cells were treated with test compounds and the induced luciferase activities were compared (Fig. 1B). Transcriptional activation by 1,25(OH)2D3 and LCA was similar to previous reports (12). Esterification of the side chain carboxyl group of LCA with methyl, ethyl, and benzyl groups drastically decreased the activity on VDR (Fig. 1B). Next, we examined the effects of LCA derivatives modified at the 3α-hydroxyl group (Fig. 1A). LCA formate and LCA acetate were able to activate VDR as efficiently as LCA at the concentration of 10 μM. LCA isobutyrate activated VDR moderately, whereas LCA hemisuccinate was not an effective VDR agonist. The data indicate that addition of a large acyl group at the 3α-hydroxyl group of LCA abolishes VDR activation. The stereochemistry, as well as the substituent of the 3-hydroxyl group, is also important for LCA activity. Iso-LCA with a 3β-hydroxyl group and ursocholanic acid with no hydroxyl group at C-3 (Fig. 1A) have little activity on VDR (Fig. 1B). Interestingly, although the effect of LCA methyl ester on VDR activation was weak, LCA acetate methyl ester was able to induce VDR activation effectively. 3-Keto-LCA, a metabolite of LCA, is another potent bile acid for VDR (12). The esterification on the side chain of 3-keto-LCA modestly decreased its activity on VDR (Fig. 1B). 6-Keto-LCA is a very weak VDR agonist, and 7-keto-LCA and 12-keto-LCA were not able to activate VDR (12). Transactivation of VDR by 3,6-diketo-LCA, 3,7-diketo-LCA, and 3,12-keto-LCA was almost absent (Fig. 1B). These data indicate that addition of a ketone moiety at position 6, 7, or 12 to LCA or 3-keto-LCA disturbs the interaction with VDR.

LCA acetate is a potent agonist for VDR

We compared VDR dose-response curves for LCA, LCA formate, LCA acetate, LCA acetate methyl ester, and 3-keto-LCA. LCA acetate activated VDR with an EC_{50} of 0.40 μM, followed in rank order by LCA formate (EC_{50} = 4.0 μM), 3-keto-LCA (6.8 μM), and LCA (12.1 μM) (**Fig. 2A**). Notably, the potency of VDR activation by LCA acetate on VDR was 30-fold greater than that of LCA.

Upon ligand binding, nuclear receptors undergo a conformational change that induces recruitment of coactivators, such as SRC-1, and dissociation of corepressors, such as N-CoR (21). To assay ligand-dependent interactions of VDR with cofactors, the receptor-interacting domains of SRC-1 and N-CoR were fused to the GAL4 DNA binding domain (14). Cotransfection of GAL4-cofactors with VDR fused to the transactivation domain of herpesvirus VP16 protein allowed for the detection of ligand-dependent cofactor interaction (15). Although there was no association between control GAL4 protein and VP16-VDR, LCA acetate at 10 μM and 1,25(OH)2D3 at 100 nM strongly induced the association of VDR with SRC-1 (Fig. 2B). The effects of LCA formate and LCA acetate methyl ester on this interaction were modest, and activation by LCA and 3-keto-LCA were weak at 10 μM concentration. LCA acetate, LCA formate, LCA acetate methyl ester, and 3-keto-LCA dissociated N-CoR from VDR as effectively as 1,25 (OH)2D3 (Fig. 2B). The effects of these LCA derivatives on N-CoR dissociation were stronger than that of LCA. Thus, LCA acetate is a potent regulator of VDR-cofactor interaction.

Next, we assessed the ability of LCA derivatives to bind

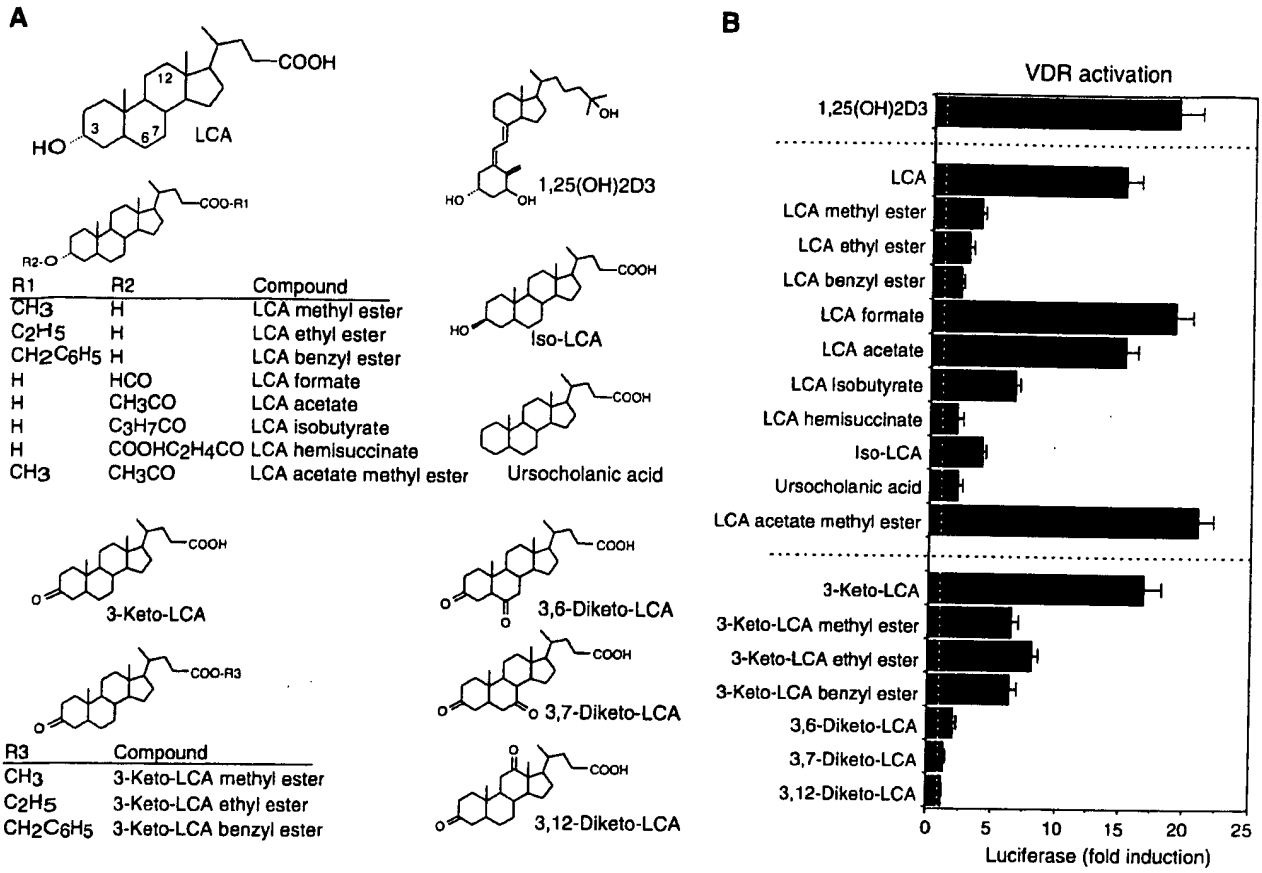

Fig. 1. Lithocholic acid (LCA) derivatives activate vitamin D receptor (VDR). A: Structures of LCA, its derivatives, and 1α,25-dihydroxyvitamin D_3 [1,25(OH)2D3] are shown. B: Activation of VDR by LCA derivatives. HEK293 cells were cotransfected with CMX-VDR and CYP3A4-ER-6x3-tk-LUC and then treated with vehicle control (ethanol), 1,25(OH)2D3 (100 nM), or bile acid derivatives (10 μM) for 24 h. Luciferase activity of the reporter is expressed as fold induction with compound treatment relative to vehicle control. The values represent means ± SD.

directly to VDR in vitro using the competitive binding assay. Isotopically labeled 1,25(OH)2D3 was incubated with in vitro translated VDR protein in the absence or presence of test compounds. The binding of labeled 1,25(OH)2D3 to VDR was competed by the addition of unlabeled 1,25(OH)2D3 (Fig. 2C). LCA acetate and LCA formate also inhibited the binding of labeled 1,25(OH)2D3 to VDR, indicating that these LCA derivatives directly bind to VDR. Competition with 3-keto-LCA and LCA was weaker than that of LCA acetate and LCA formate. Interestingly, although LCA acetate methyl ester showed enhanced activation of VDR compared with 3-keto-LCA in the luciferase reporter assay, as shown Fig. 2A, its direct interaction with VDR protein was weaker than those of LCA and 3-keto-LCA (Fig. 2C). LCA acetate did not inhibit the binding of labeled estradiol to ERα (data not shown). Taken together, these data indicate that LCA acetate activates VDR by direct binding.

LCA acetate is not a potent agonist for other bile acid receptors

The ligand binding domains of various nuclear receptors were fused to the DNA binding domain of the yeast transcription factor GAL4 to examine the effect of LCA acetate on these receptors. The GAL4-chimera receptors were cotransfected with a GAL4-responsive luciferase reporter into HEK293 cells (15). Because this reporter is activated only by GAL4-chimera receptors, the potentially confounding effects of endogenous receptors are eliminated. LCA acetate at 30 μM induced the activation of GAL4-VDR (**Fig. 3A**). It induced weak activation of FXR but was not effective on TRα, RARα, PPARα, PPARδ, PPARγ, LXRα, CAR, RXRα, or ERα. FXR has been previously shown to respond to various bile acids, such as chenodeoxycholic acid and deoxycholic acid (12, 15). Next, we determined FXR dose-response curves for LCA derivatives modified at position 3. As reported previously (15), chenodeoxycholic acid was a potent FXR agonist (Fig. 3B). Interestingly, ursocholanic acid and iso-LCA, which were not effective on VDR (Fig. 1B), strongly induced the activation of FXR (Fig. 3B). LCA formate and LCA acetate, as well as LCA, were weak FXR agonists. PXR was reported to respond to high concentrations of LCA (10, 11). To examine the effects of LCA derivatives on PXR, we transfected VDR or PXR expression vectors with a reporter containing a CYP3A4 element, which can be activated by both re-

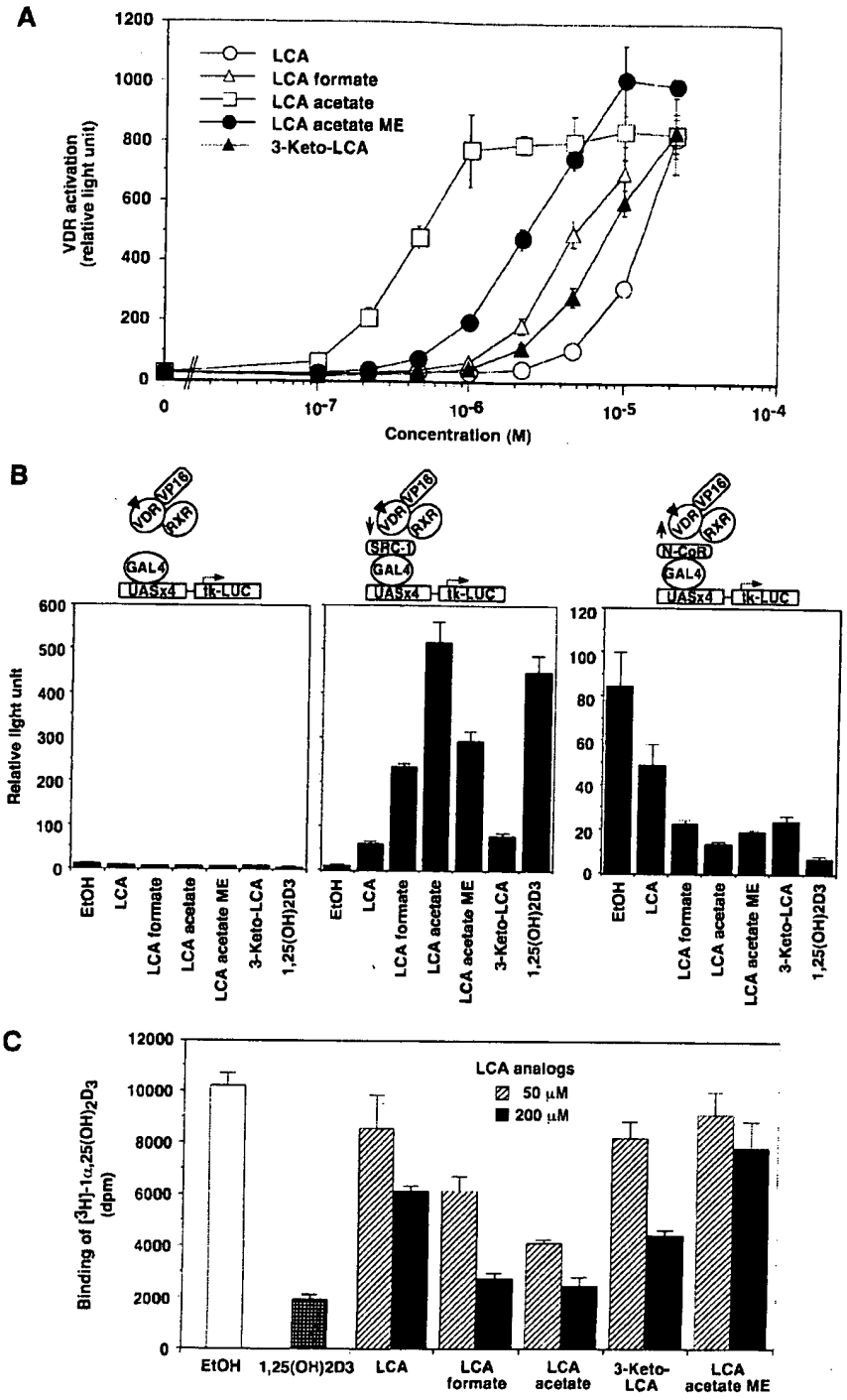

Fig. 2. LCA acetate is a potent VDR agonist. A: Concentration-dependent activation of VDR by LCA acetate and its related compounds. HEK293 cells were cotransfected with CMX-VDR and CYP3A4-ER-6x3-tk-LUC reporter and treated with several concentrations of LCA, LCA formate, LCA acetate, LCA acetate methyl ester (LCA acetate ME), and 3-keto-cholanic acid (3-keto-LCA) for 16 h. B: Interactions of VDR with steroid receptor coativator-1 (SRC-1) and nuclear receptor corepressor (N-CoR) induced by LCA acetate and its related compounds. HEK293 cells were cotransfected with GAL4 control vector or GAL4-chimera vectors for SRC-1 or N-CoR, in combination with VP16-VDR and MH100(UAS)x4-tk-LUC reporter, and were treated with ethanol (EtOH) control, 10 μM LCA acetate, or related bile acids. C: Direct binding of LCA acetate to VDR. In vitro translated VDR proteins were incubated with 1 nM [^{3}H]1,25(OH)2D3 in the presence or absence of nonradioactive 10 nM 1,25(OH)2D3 or 50 μM or 200 μM bile acid derivatives. The values represent means ± SD.

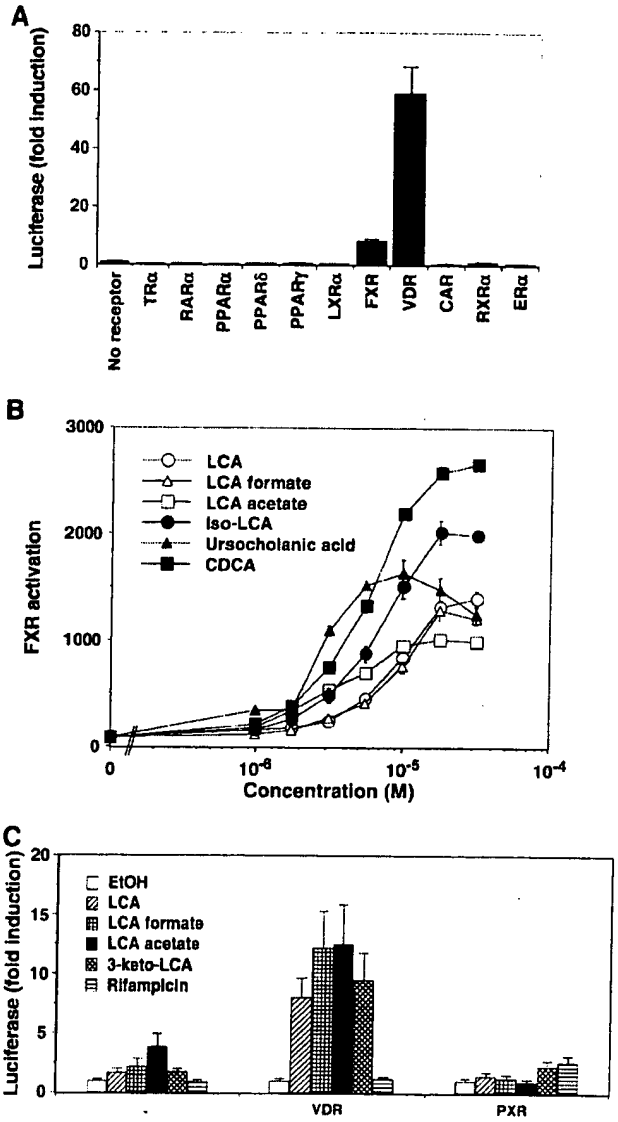

Fig. 3. LCA acetate is a selective agonist for VDR. A: Receptor-specific activation by LCA acetate. GAL4-chimera receptors for various nuclear receptors were expressed with MH100(UAS)x4-tk-LUC reporter in HEK293 cells and assayed for activation by 30 μM LCA acetate. Luciferase activity of the reporter is expressed as fold induction with compound treatment relative to vehicle control. PPAR, peroxisome proliferator-activated receptor. B: Concentration-dependent activation of farnesoid X receptor (FXR) by LCA acetate and its related compounds. HEK293 cells were cotransfected with CMX-FXR and IR-1x3-tk-LUC reporter and treated with several concentrations of LCA, LCA formate, LCA acetate, iso-LCA, ursocholanic acid, or chenodeoxycholic acid (CDCA). C: Comparative response of VDR and pregnane X receptor (PXR) to LCA acetate in liver HepG2 cells. HepG2 cells were transfected with CMX control vector (−), CMX-VDR, or CMX-PXR with CYP3A4-ER-6x3-tk-LUC and treated with vehicle control [ethanol (EtOH)], 30 μM LCA, LCA formate, LCA acetate, 3-keto-LCA, or rifampicin. The values represent means ± SD.

ceptors (12). Liver-derived HepG2 cells were used for this experiment, because PXR activation is cell type dependent (data not shown). In the absence of transfected receptors, the luciferase activity was increased by addition of the LCA derivatives (Fig. 3C). This effect may be derived from endogenous receptors such as VDR. LCA acetate and LCA formate strongly induced the activity of transfected VDR, indicating that these LCA derivatives activate VDR in HepG2 cells. The PXR agonist rifampicin did not activate VDR. When HepG2 cells were cotransfected with PXR, rifampicin and 3-keto-LCA increased the reporter activity, but LCA acetate and LCA formate were not effective PXR ligands (Fig. 3C). These findings indicate that LCA acetate is selective for VDR activation.

Effect of VDR mutation on LCA acetate response

To elucidate the structure-activity relationship of LCA acetate and VDR, we examined the effects of LCA acetate on the activation of several VDR mutants. Wild-type GAL4-VDR and several alanine mutants, Y143A, S237A, S275A, S278A, W286A, and H305A, were introduced into HEK293 cells and activation by LCA, LCA acetate, and 1,25(OH)2D3 were compared (**Fig. 4A**). According to the crystal structure of the VDR-1,25(OH)2D3 complex (22), Y143 and S278 interact with the 3β-hydroxyl group of 1,25(OH)2D3, S237 hydrogen bonds with the 1α-hydroxyl group, H305 coordinates the 25-hydroxyl group, and S275 and W286 mediate hydrophobic interaction with 1,25(OH)2D3. The Y143A and W286A mutations inhibited activation by LCA acetate, LCA, and 1,25(OH)2D3. The effect of S237A was modest on LCA, LCA acetate, and 1,25(OH)2D3 activity. Whereas S275A and S278A almost abolished the activity of LCA, LCA acetate and 1,25(OH)2D3 still activated these mutants. Interestingly, although H305A had significant effects on the activity of LCA and 1,25(OH)2D3, this mutation had little effect on the activity of LCA acetate. Thus, LCA acetate is similar to 3-keto-LCA in its ability to activate VDR-H305A (13). In a previous study, we found that the VDR-S278V mutant is activated by 1,25(OH)2D3 but not by LCA, whereas VDR-S237M can respond to LCA but not to 1,25(OH)2D3 (14). We next examined the effects of LCA acetate on these mutants (Fig. 4B). The S237M mutation weakly affected the activity of LCA acetate as well as that of LCA. S278V drastically decreased LCA acetate activity. Based on these findings, we modeled LCA acetate in the VDR ligand binding domain (Δ165–215) (PDB #1DB1) using FlexX software (13). In contrast to the LCA docking model, the side chain of LCA acetate directs to the β-turn site (Fig. 4C, left panel) (13). The oxygen of the side chain carboxyl group and the carbonyl oxygen of the 3-*O*-acetyl acetate group nearly overlap with the 3β-hydroxyl oxygen and 25-hydroxyl oxygen, respectively, of 1,25(OH)2D3 in the crystal structure of VDR-1,25(OH)2D3 (Fig. 4C, right panel). The proximity of these amino acid residues to hydrogen bond acceptors within 1,25(OH)2D3 may be responsible for the strong activity of LCA acetate on VDR.

Induction of VDR target genes by LCA acetate in intestinal cells

VDR is highly expressed in intestinal mucosa cells and regulates the expression of genes involved in calcium homeostasis and bile acid metabolism (1, 12, 23). We investi-

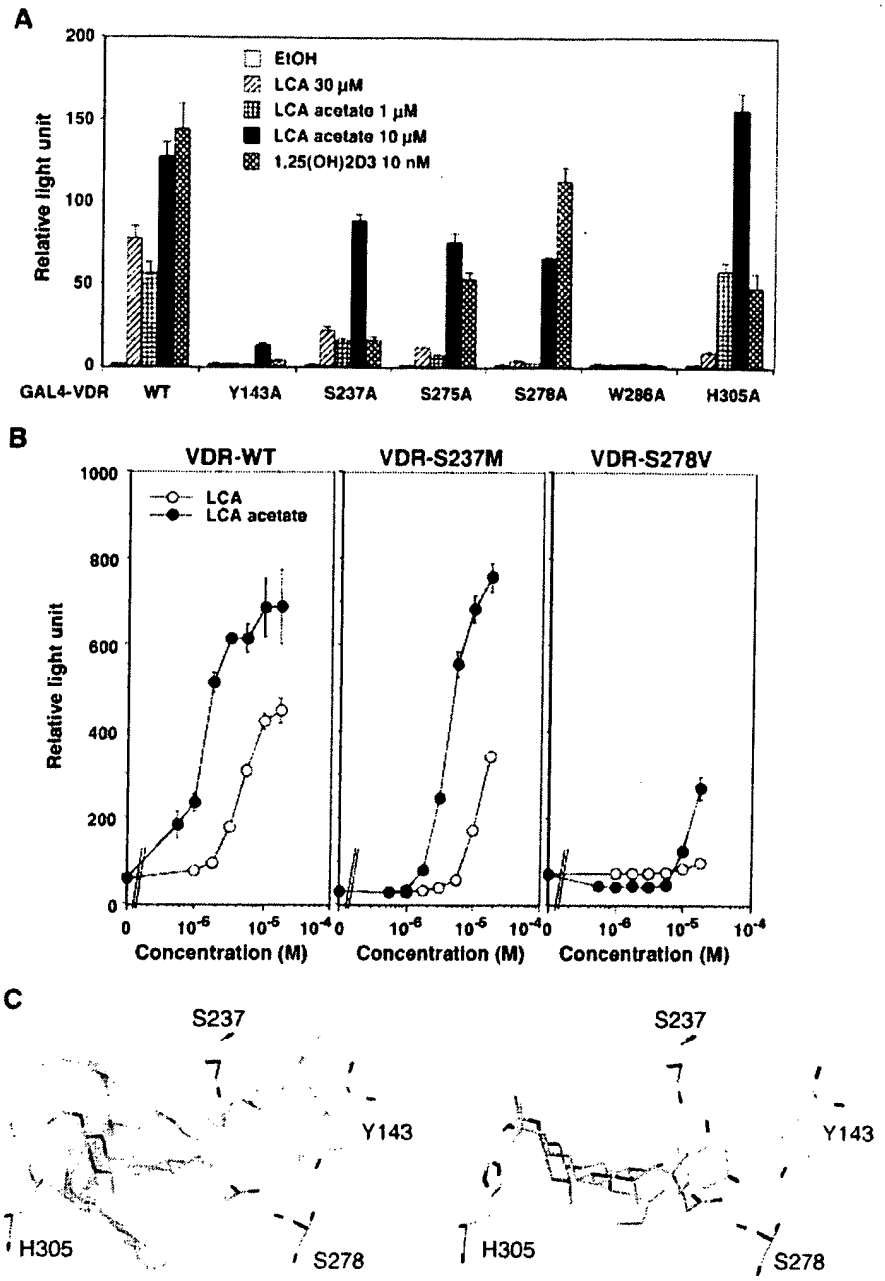

Fig. 4. Structure-function analysis of LCA acetate and VDR. A: Activation of VDR or its mutants by LCA acetate. GAL4-VDR and alanine mutants (Y143A, S237A, S275A, S278A, W286A, and H305A) were cotransfected with MH100(UAS)x4-tk-LUC reporter in HEK293 cells and treated with vehicle control [ethanol (EtOH)] or the indicated concentrations of test compounds. WT, wild type. B: Dose response of VDR S237M and S278V mutants for LCA acetate. HEK293 cells were cotransfected with GAL4-VDR, GAL4-VDR-S237M, or GAL4-VDR-S278V with MH100(UAS)x4-tk-LUC reporter in HEK293 cells. The values represent means ± SD. C: Docking model of VDR interaction with LCA acetate. Left panel: The side chain carboxyl group is directed to the β-turn site interacting with S278. The Connolly channel surface of the VDR ligand binding pocket is shown in translucent gray. Right panel: Overlay of LCA acetate (yellow) and 1,25(OH)2D3 (gray) accommodated in the VDR ligand binding pocket.

gated the ability of LCA acetate to activate endogenous VDR target genes in intestinal cells. Colon cancer-derived SW480 cells were incubated with LCA, LCA acetate, 1,25(OH)2D3, chenodeoxycholic acid, or rifampicin, and the expression of VDR target genes, including CYP24A1, CYP3A4, CaT1, and E-cadherin, was examined. CYP24A1 and CaT1 are involved in calcium homeostasis and CYP3A4 metabolizes LCA (1, 24). E-cadherin was reported to be

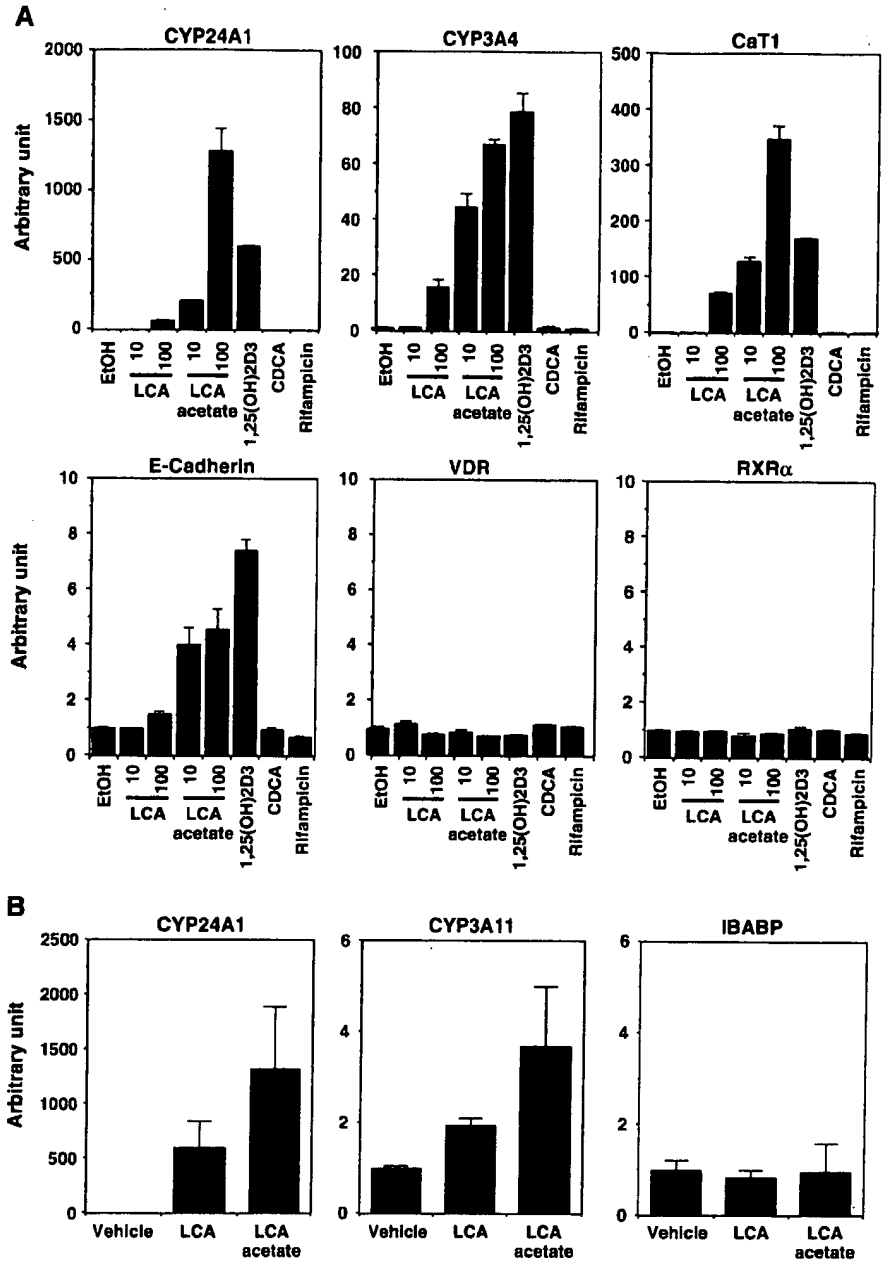

Fig. 5. Induction of VDR target genes by LCA acetate in intestinal cells. A: LCA acetate induced VDR target genes more effectively than LCA in colon cancer-derived SW480 cells. Cells were treated with vehicle control [ethanol (EtOH)], 10 or 100 μM LCA or LCA acetate, 100 nM 1,25(OH)2D3, 100 μM chenodeoxycholic acid (CDCA), or 30 μM rifampicin for 24 h. Quantitative real-time PCR from mRNA for CYP24A1, CYP3A4, CaT1, E-cadherin, VDR, and retinoid X receptor α (RXRα) was performed. B: LCA acetate increased VDR target genes in mouse intestine more effectively than LCA. Mice were orally administrated with 200 mg/kg LCA or LCA acetate. Twelve hours after administration, total RNA was extracted from intestinal mucosa and quantitative real-time PCR from mRNA for CYP24a1, Cyp3a11, and ileal bile acid binding protein (IBABP) was performed. The values represent means ± SD.

associated with cell growth inhibition induced by 1,25 (OH)2D3 (25). As shown in **Fig. 5A**, 1,25(OH)2D3 induced the expression of CYP24A1, CYP3A4, CaT1, and E-cadherin. LCA acetate induced these VDR target genes more effectively than LCA, indicating that LCA acetate acts as a potent VDR agonist in colon cancer cells. There were no changes in the expression of VDR and RXRα, which forms a heterodimer with VDR, after treatment with the VDR ligands. The FXR agonist chenodeoxycholic acid and the PXR agonist rifampicin were not able to in-

duce the expression of these genes, although PXR was reported to be involved in CYP3A4 gene regulation (11). The inability of PXR agonist to increase gene expression is likely attributable to the fact that PXR is not expressed in SW480 cells (data not shown). Next, we examined the expression of VDR target genes in vivo. Mice were orally administered LCA or LCA acetate, and the expression of intestinal Cyp24a1 and Cyp3a11 genes was evaluated. Both LCA and LCA acetate increased the mRNA expression of Cyp24a1 and Cyp3a11 significantly, LCA acetate being more effective than LCA (Fig. 5B). LCA and LCA acetate did not induce the FXR target IBABP gene expression (Fig. 5B). These data indicate that LCA acetate is a potent agonist for endogenous VDR in intestinal cells.

LCA acetate induces the differentiation of monoblastic leukemia cells

1,25(OH)2D3 is known as an inducer of myeloid leukemia differentiation (1). We examined the effects of LCA acetate on the growth and differentiation of human monoblastic leukemia THP-1 cells. 1,25(OH)2D3 inhibited the proliferation of THP-1 cells and enhanced NBT-reducing activity, a differentiation marker of myeloid leukemia cells, as reported previously (26). LCA acetate inhibited cell proliferation more effectively than LCA and 3-keto-LCA (**Fig. 6A**), and it induced NBT-reducing activity in the cells. In contrast, LCA and 3-keto-LCA were not able to induce this activity even at concentrations that completely inhibit cell proliferation (Fig. 6B). Untreated THP-1 cells have large nuclei with visible nucleoli and basophilic cytoplasmic staining. LCA acetate induced a concentration-dependent increase in the percentage of differentiated cells (Fig. 6C). In cells treated with LCA acetate, the nuclei were condensed, nucleoli were no longer apparent, and the cytoplasm appeared gray, indicating monocytic differentiation (Fig. 6C). Esterase activity, a functional marker of monocytic differentiation, was also induced by LCA acetate (Fig. 6D). LCA and 3-keto-LCA did not induce morphological and functional differentiation of THP-1 cells. LCA acetate increased the expression of surface makers, such as CD11b and CD14, as effectively as 1,25(OH)2D3 (Fig. 6E). Therefore, the VDR agonist LCA acetate is a potent inducer of monocytic differentiation in THP-1 leukemia cells.

DISCUSSION

In this study, we found that the modification of the 3α-hydroxyl group of LCA increases the transactivation activity and selectivity on VDR. Structure-function relationship analysis of the VDR-LCA interaction using several VDR mutants shows that the side chain of LCA faces H12 of the receptor and 3-keto-LCA is directed toward the β-turn site (13). As shown in Fig. 1, esterification of the side chain carboxyl group of LCA abolished VDR activation. However, in 3-keto-LCA, the corresponding esterifications had only moderate effects. This may be ascribed to the opposing docking modes of LCA and 3-keto-LCA. The LCA derivatives modified at position 3, such as LCA formate and LCA acetate, have stronger activity than LCA (Figs. 1, 2). The docking model shown in Fig. 4 indicates that LCA acetate is accommodated in the VDR ligand binding pocket in the same manner as 3-keto-LCA and that LCA acetate can form hydrogen bonds with the same amino acid residues that coordinate 1,25(OH)2D3 binding. LCA acetate methyl ester has much stronger activity than LCA methyl ester. This may be attributable to different docking modes of these two LCA esters. LCA acetate and LCA can activate VDR-S237M (Fig. 4B), which does not respond to 1,25 (OH)2D3 (14). S237 is located in H3 and may mediate allosteric communication with the cofactor interaction surface. These findings suggest the possibility that LCA acetate induces an alternative conformation in VDR, which results in differential cofactor recruitment and selective physiological function. Further study is required to elucidate the structure-function relationship of VDR and LCA derivatives such as LCA acetate.

FXR is activated by both primary bile acids (chenodeoxycholic acid and cholic acid) and secondary bile acids (LCA and deoxycholic acid) (15, 27, 28). In contrast, VDR responds to only LCA and its derivatives (12). In the previous study, 6-keto-LCA was identified as a selective ligand for VDR, but its activity was very weak (12). The potent VDR agonist LCA acetate activated FXR to low levels, similar to the weak FXR agonist LCA, and much more weakly than chenodeoxycholic acid (Fig. 3B). In HepG2 cells, chenodeoxycholic acid induced the expression of the BSEP gene, which is an FXR target (9), but LCA and LCA acetate were not effective in its induction, although LCA acetate increased the VDR target CYP24A1 expression (see supplementary data online). Although LCA and 3-keto-LCA were agonists for PXR at high concentrations, LCA acetate did not activate PXR (Fig. 3C). These data indicate that LCA acetate is a selective agonist for VDR. Interestingly, although iso-LCA and ursocholanic acid were not able to activate VDR, they were more potent FXR agonists than LCA. Recently, crystal structures of FXR and PXR have been reported (29–31). Mutational analysis of FXR and PXR should be useful in elucidating the structure-function relationship of these LCA derivatives and in the development of selective ligands for the bile acid receptors VDR, FXR, and PXR.

Vitamin D has been identified as a protective agent against the development of colorectal cancer (32). Epidemiological analysis revealed that solar exposure, which results in vitamin D production in the skin, or vitamin D uptake reduces the incidence of colorectal cancer (32). Protective effects of vitamin D in colon carcinogenesis are mediated through its receptor VDR. VDR activation induces the expression of genes involved in growth inhibition, differentiation, and apoptosis (1, 33). In contrast to vitamin D, the secondary bile acid LCA is considered to be a promoter of colon carcinogenesis (34). LCA induces DNA strand breaks, forms DNA adducts, inhibits DNA repair enzymes, and can promote colon cancer in rodent models (35). CYP3A was reported to detoxify LCA to a nontoxic hyodeoxycholic acid and is a VDR target gene

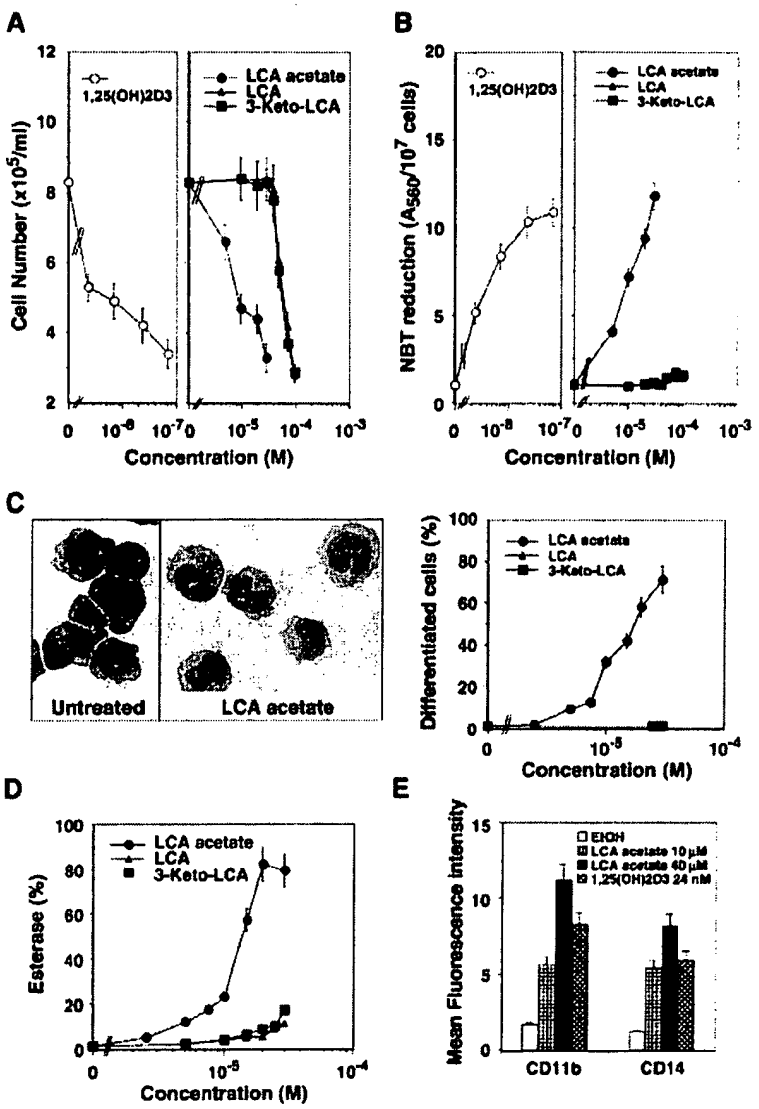

Fig. 6. Effects of LCA acetate on growth and differentiation of human myeloid leukemia THP-1 cells. A: Growth inhibition of THP-1 cells by 1,25(OH)2D3 and LCA acetate. B: Induction of nitroblue tetrazolium (NBT)-reducing activity in THP-1 cells by 1,25(OH)2D3 and LCA acetate. THP-1 cells were treated with 1,25(OH)2D3, LCA acetate, LCA, or 3-keto-LCA for 4 days. C: LCA acetate induces the morphological differentiation of THP-1 cells. Cells were treated with LCA acetate, LCA, or 3-keto-LCA for 6 days, and differentiated cells shown in the left panel were counted. D: LCA acetate induces monocyte-specific esterase activity. Cells were treated with LCA acetate, LCA, or 3-keto-LCA for 6 days. E: LCA acetate increases the expression of CD11b and CD14 surface antigens. Cells were treated with LCA or 1,25(OH)2D3 for 4 days and CD11b and CD14 expression was examined using monoclonal antibodies and flow cytometry. EtOH, ethanol. The values represent means ± SD.

(11, 36). By binding to VDR, 1,25(OH)2D3 and LCA induce CYP3A expression in the intestine. VDR may serve as a sensor for LCA and function to protect intestinal mucosa from its harmful effects. Recently, a significant correlation between a VDR polymorphism and colorectal cancer risk was reported in a Singapore Chinese population (37). These findings suggest that VDR functions as an anticancer factor and indicate that it is a promising molecular target for chemoprevention against colorectal cancer.

Clinical trials of vitamin D and its analogs have been unsuccessful because of their hypercalcemic activities (38). Structure-function analysis of vitamin D analogs suggests that 1,25(OH)2D3 and its analogs also induce nongenomic VDR actions and that adverse effects are at least partly attributable to nongenomic mechanisms (5, 39). Ligand-dependent dissociation of nongenomic from genomic activity was reported for the estrogen receptor (40). An estrogen receptor ligand, pyrazole, induced the transactivation of an estrogen receptor target gene but had weak nongenomic activity, whereas another ligand, es-

tren, induced strong nongenomic action of the estrogen receptor without altering gene expression. There has been no reported physiological correlation between bile acids and intestinal calcium absorption, suggesting that LCA or its derivatives may relatively induce genomic actions in the intestine, such as bile acid metabolism and cell growth control, without inducing hypercalcemia. LCA acetate induced VDR target genes via genomic action, including the LCA-detoxifying enzyme CYP3A, in colon cancer cells and mouse intestines more effectively than LCA (Fig. 4). Nongenomic action of bile acids and derivatives should be further investigated. The development of more potent LCA derivatives that are nontoxic and less hypercalcemic should be useful for chemoprevention against colon carcinogenesis.

1,25(OH)2D3 was found to induce the differentiation of mouse myeloid leukemia M1 cells more than 20 years ago (41). Treatment with 1,25(OH)2D3 or 1α-hydroxyvitamin D_3, which is rapidly metabolized to 1,25(OH)2D3, was reported to prolong survival in mice inoculated with M1 leukemia cells (3). The differentiation-inducing effects of 1,25(OH)2D3 were also demonstrated in human leukemia cells (42, 43). However, the molecular mechanisms of differentiation induced by 1,25(OH)2D3 have not been elucidated. We found that the potent VDR agonist LCA acetate was able to induce the differentiation of human monoblastic leukemia THP-1 cells at concentrations that induce VDR activation (Fig. 6). LCA and 3-keto-LCA inhibited proliferation but did not induce differentiation. The growth-inhibiting activity of these bile acids may be attributable to their cytotoxic effects. Zimber et al. (44) reported that bile acids, including deoxycholic acid, chenodeoxycholic acid, and LCA, induced the differentiation of human promyelocytic leukemia HL-60 cells. We did not observe differentiation-inducing activity of these bile acids in HL-60 cells (data not shown). This is probably because of differences between subclones of leukemia cell lines, which could affect sensitivity to the compounds. Regardless, LCA acetate did induce differentiation markers in HL-60 cells (data not shown). These findings indicate that LCA acetate is a more effective inducer of leukemia differentiation than bile acids such as LCA and chenodeoxycholic acid. Zimber et al. (45) reported that LCA alone did not induce the differentiation of THP-1 cells but that it enhanced the response to all-*trans*-retinoic acid, which is a potent differentiation inducer of myeloid leukemia cells. The combinational effects of LCA acetate and other differentiation inducers are now under investigation. The protein kinase C inhibitor sphingosine decreased the NBT-reducing activity induced by deoxycholic acid and chenodeoxycholic acid in HL-60 cells but did not alter the response to LCA (44), suggesting that the effect of LCA is mediated by mechanisms distinct from those used by deoxycholic acid and chenodeoxycholic acid. Expression of some VDR target genes was increased in THP-1 cells after treatment with LCA acetate (data not shown). The data indicate that LCA acetate functions as a VDR agonist in leukemia cells and induces cell differentiation. Further studies are required to elucidate the precise mechanisms of LCA acetate- and 1,25(OH)2D3-induced leukemia cell differentiation.

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FULL TEXT OF CASES (USPQ2D)
All Other Cases

Noelle v. Lederman, 69 USPQ2d 1508 (CA FC 2004)

69 USPQ2D 1508
Noelle v. Lederman

U.S. Court of Appeals Federal Circuit

No. 02-1187
Decided January 20, 2004

Headnotes

PATENTS

[1] Patentability/Validity — Specification — Written description (§115.1103)

Patent claim directed to any antibody which is capable of binding to particular antigen has sufficient support in written description that discloses "fully characterized" antigens; thus, if applicant has disclosed fully characterized antigen, either by structure, formula, chemical name, or physical properties, or by depositing protein in public depository, then applicant can claim antibody by its binding affinity to that described antigen.

[2] Patentability/Validity — Date of invention — In general (§115.0401)

Patentability/Validity — Specification — Written description (§115.1103)

Applicant's claims to human form of "CD40CR" antibody in continuation application

are not supported by written description in prior application, even though earlier application stated that human CD40CR antibody binds to human CD40CR antigen, since prior application, which only described mouse CD40CR antigen, did not disclose "fully characterized" human CD40CR antigen, and since application therefore attempted to define one unknown by its binding affinity to another unknown; moreover, applicant cannot claim genus form of CD40CR antibody from description of mouse CD40CR antigen, since patentee of biotechnological invention cannot necessarily claim genus after describing only limited number of species, in that there may be unpredictability in results obtained from species other than those specifically enumerated.

[3] Practice and procedure in Patent and Trademark Office — Interference — Rules and rules practice (§110.1704)

Board of Patent Appeals and Interferences properly applied "two-way"test in finding that there was no interference-in-fact between senior party's patent and junior party's application, since board determined that person of skill in relevant art would have lacked reasonable expectation of success in obtaining senior party's claimed human form of "CD40CR" antibody if provided with junior party's claimed mouse CD40CR antibody and screening techniques cited by junior party; even though board was not required to conduct second prong of test in order to find no interference-in-fact, it nonetheless found that person of skill in art would have lacked reasonable expectation of success in obtaining junior party's mouse CD40CR antibody if provided with senior party's claimed human CD40CR antibody and same screening methods.

[4] Practice and procedure in Patent and Trademark Office — Interference — Rules and rules practice (§110.1704)

Patentability/Validity — Obviousness — Relevant prior art — Particular inventions (§115.0903.03)

Board of Patent Appeals and Interferences correctly found no interference-in-fact between parties' claims to human form of "CD40CR" antibody, since board properly refused to consider methods of antigen isolation that were found in specification of junior party's prior application, but were not disclosed in junior party's claims, and since, given state of prior art at time of junior party's application, person of ordinary skill in art would not have had reasonable likelihood of success in isolating human CD40CR antibodies from mouse CD40CR antigen and its antibodies disclosed in junior party's application.

Particular Patents

Particular patents — Chemical — Human antibody

5,474,771, Lederman, Chess, and Yellin, murine monoclonal antibody (5c8) recognizes a human glycoprotein on the surface of T-lymphocytes, compositions containing same, finding of no interference-in-fact with application no. 08/742,480, in interference no. 104,415, affirmed.

Case History and Disposition

Appeal from the U.S. Patent and Trademark Office, Board of Patent Appeals and Interferences.

Patent interference proceeding (no. 104,415) between Randolph J. Noelle (application no. 08/742,480), junior party, and Seth Lederman, Leonard Chess, and Michael J. Yellin (patent no. 5,474,771), senior party. Junior party appeals from finding of no interference-in-fact. Affirmed.

Attorneys:

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James F. Haley Jr., Margaret A. Pierri, Jane T. Gunnison, and Stanley Den-Kua Liang, of Fish & Neave, New York, N.Y.; John P. White, of Cooper & Dunham, New York, for appellees.

Judge:

Before Clevenger, Bryson, and Gajarsa, circuit judges.

Opinion Text

Opinion By:
Gajarsa, J.

This is an appeal from an interference proceeding involving the claims of United States Patent Application Serial No. 08/742,480 (the "'480 application") and United States Patent No. 5,474,771 (the "'771 patent"). Randolph J. Noelle ("Noelle") is the inventor named on the '480 application. Seth Lederman, Leonard Chess, and Michael J. Yellin (collectively "Lederman") are the inventors named on the '771 patent. Noelle appeals the decision of the United States Patent and Trademark Office,

Board of Patent Appeals and Interferences ("Board"), finding no interference-in-fact between the '480 application and the '771 patent and rejecting claims 51, 52, 53, 56, 59, and 60 of the '480 application pursuant to 35 U.S.C. §102(b) (2000). *Noelle v. Lederman*, Interference No. 104,415 (Bd. Pat. App. & Int. Oct. 19, 2001). Because the decision of the Board is supported by substantial evidence and is not contrary to law, we affirm.

BACKGROUND

A. *Antibodies*

This case relates to antibodies and their role in the immune response system. A vertebrate's immune system serves to identify and destroy foreign invading organisms and neutralize the toxic molecules they produce. Antibodies, which are proteins also referred to as immunoglobulins ("Ig"), serve to designate foreign particles, broadly referred to as antigens, for destruction by other components of the immune system such as lymphocytes.1 Lymphocytes, otherwise known as white blood cells, produce antibodies and destroy antigens. T-cells and B-cells are the two types of lymphocytes needed for antibody production. One specific type of T-cell is the helper T-cell. Helper T-cells recognize antigens and then induce B-cells to produce antibodies through a series of events. First the helper T-cell is activated after it recognizes an antigen. Once activated, the helper T-cell activates the B-cell by a combination of binding with the B-cell and secreting signaling molecules. Once the B-cell is activated, it differentiates,2 proliferates, and produces antibodies specific to a particular antigen. The antibodies then circulate in the bloodstream and permeate other bodily fluids, where they bind to the antigen, thereby flagging it for destruction.

The present interference involves competing claims to an antibody ("CD40CR antibody") that represses the cell-to-cell signaling interaction between helper T-cells and B-cells. CD40CR antigen 3 is found on activated, but not resting, helper T-cells. CD40CR antigen acts as a "key" to unlock a protein ("CD40") located on the surface of resting B-cells. Once CD40CR antigen and CD40 bind, the B-cell begins down the pathway to differentiation, proliferation, and antibody production. The CD40CR antibody

binds to the CD40CR antigen located on the T-cell surface, thereby inhibiting its ability to bind to the CD40 receptor located on the resting B-cell. B-cells cannot then become activated, thereby preventing the B-cell from producing antibodies. CD40CR antibodies are useful for treating a hyperactive immune system that causes allergic reactions and autoimmune diseases.

B. *The Interference*

Noelle's '480 application was filed November 1, 1996. The '480 application is a continuation of application Serial No. 08/338,975 ("the '975 application"), filed November 14, 1994, which is in turn a continuation of application Serial No. 07/835,799 ("the '799 application"), filed on February 14, 1992. The claims of Noelle's '480 application are directed to the genus, murine ("mouse"), chimeric ("hybrid"), humanized, and human forms of the CD40CR monoclonal antibody. Noelle also claims the hybridomal 4 cell lines that produce the CD40CR antibody.

Lederman's '771 issued patent has an effective filing date of November 15, 1991. Lederman's '771 patent describes and claims the human form of CD40CR monoclonal antibody (the "5c8 antibody"). The 5c8 antibody binds to "the 5c8 antigen located on the surface of activated T cells and thereby inhibits T cell activation of B cells." Also, Lederman claims a hybridomal cell line created to produce monoclonal antibody 5c8.

On September 3, 1999, an interference was declared by the United States Patent and Trademark Office ("USPTO") between the issued claims of Lederman's '771 patent and Noelle's '480 application. Noelle was designated the junior party and Lederman was designated the senior party based on their effective filing dates. The USPTO established only one count in the interference. The count reads as follows:

> The monoclonal antibody of claim 1 of 5,474,771 or the monoclonal antibody of claim 42 or claim 51 of 08/742,480.
> Claim 1 of Lederman's '771 patent reads as follows:
> A monoclonal antibody, which specifically binds and forms a complex with the 5c8 antigen located on the surface of activated T cells and thereby inhibits T cell activation of B cells, the 5c8 antigen being an antigen to which monoclonal antibody 5c8 (ATCC Accession No. HB 10916) specifically binds.
> Claim 42 of Noelle's '480 application reads as follows:
> A monoclonal antibody or fragment thereof which specifically binds to an antigen expressed on activated T cells, wherein said antigen is specifically bound by the monoclonal antibody secreted by hybridoma MR1 which hybridoma has been deposited and accorded ATCC Accession No. HB 11048.
> Claim 51 of Noelle's '480 application reads as follows:
> A monoclonal antibody or fragment thereof which specifically binds CD40CR.
> Claim 52 of Noelle's '480 application reads as follows:
> The monoclonal antibody or fragment of Claim 51, wherein said CD40CR is expressed

by activated human T cells.

For sake of the simplicity, Claim 1 of Lederman's '771 patent and Claim 52 of Noelle's '480 application will be referred to as claims to the "human" form of CD40CR antibody. Claims 42 and 51 of Noelle's '480 application will be referred to as claims to the "mouse" and "genus"forms of CD40CR antibody, respectively.

On June 28, 2001 the Board held a hearing to dispose of the parties' preliminary motions. Lederman moved to have Noelle's claims rejected and sought to redefine the count. Likewise, Noelle also sought to have the count redefined. The Board denied Lederman's motions for judgment against Noelle's mouse claims for lack of written description, lack of enablement, and indefiniteness. *See* 35 U.S.C. §112 (2000). The Board found that Lederman had failed to demonstrate that the mouse claims in Noelle's '480 application failed to comply with 35 U.S.C. §112, paragraphs (1) and (2), as of November 1, 1996, the date Noelle filed his '480 application. The Board, however, determined that the human and genus claims in Noelle's '480 application failed to comply with the written description requirement pursuant to 35 U.S.C. §112, paragraph (1), as of February 14, 1992, the date Noelle filed the previous '799 application. The Board made a detailed analysis of this court's precedent pertaining to the doctrine of written description, focusing on the holding from *Regents of the University of California v. Eli Lilly & Co.* that an "adequate written description of a DNA sequence claim requires a precise definition, such as structure, formula, chemical name, or physical properties." 119 F.3d 1559, 1566 [43 USPQ2d 1398] (Fed. Cir. 1997). The Board analogized the DNA claims from *Regents* to the antibodies in Noelle's application. Accordingly, the Board held that Noelle's claims regarding the genus and human claims from the '480 application lacked written description support in the specification of Noelle's earlier '799 application because Noelle failed to describe any structural features of the human or genus antibodies or antigens. In other words, the Board found that the claims covering the genus and human antibodies constituted new matter because they lacked adequate written description in Noelle's earlier '799 application. The Board did not reject the claims, but rather denied them the benefit of the earlier filing date of Noelle '799.

Next, the Board addressed the implication of finding a lack of written description for the genus and human claims in Noelle's '480 application. The Board determined that the claims to the human and genus forms of CD40CR antibody in Noelle's '480 application were anticipated by either Lederman '771, which claims priority to U.S. Application 07/

792,728, filed November 15, 1991, or Armitage 5,961,974 (the "'974 patent"), which claims priority to U.S. applications 07/783,707 and 07/805,723 filed October 25, 1991, and December 5, 1991, respectively. Noelle had not attempted to distinguish his human and genus claims from the prior art and had conceded that Lederman '771 and Armitage '974 would anticipate those claims if the '480 application were not afforded the earlier filing date of Noelle's '799 application. Thus, the Board found the genus and human claims of Noelle's '480 application to be anticipated under 35 U.S.C. §102(b) by the two forms of prior art and, as a result, rejected the claims to the human and genus forms of CD40CR antibodies and their respective cell lines pursuant to 37 C.F.R. § 1.641.

On October 19, 2001, the Board ruled on the motions remaining from the previous hearing. The Board had determined in its previous hearing that the deferred motions were essentially requests to decide whether an interference-in-fact existed between the two parties' claims. Lederman then withdrew his pending motions and filed a new motion requesting that the Board find no interference-in-fact.

The Board concluded from the evidence submitted that there was no interference-in-fact. The Board reasoned that a person of ordinary skill in the art lacked a reasonable expectation of success of obtaining the other party's claimed invention given the state of the art at the time. The Board noted three different methods disclosed in Noelle's '480 specification by which a person of ordinary skill in the art could have isolated the human form of the CD40CR antibody given the mouse version of the CD40CR antibody. Dr. Edward A. Clark, Noelle's expert, declared that a person skilled in the art would have had a reasonable expectation of success in isolating human CD40CR antibody by utilizing the methods disclosed in Noelle's specification.

First, Clark testified that human CD40CR antibody could be isolated by immunizing a host with human CD40CR antigen expressing cells or cell lines and selecting the antibody to the CD40CR antigen by functional or competition binding with CD40-Ig.5 Next, Clark suggested methods of making and isolating antibodies using affinity purified human CD40CR antigen. Last, Dr. Clark declared that one skilled in the art could use the mouse CD40CR antibody or CD40-Ig to clone CD40CR antigen DNA using a method known as expression cloning.

The Board found that one skilled in the art would not have had a reasonable expectation of success of isolating human CD40CR antibodies given the mouse form of CD40CR antigen. At the outset, the Board reasoned that any reference to Noelle's own specification as prior art was improper because the specifications underlying the respective claims cannot be considered "prior art" and an interference-in-fact analysis requires the comparison between the parties'claims, not their specifications. *In re Vaeck*, 947 F.2d 488, 493 [20 USPQ2d 1438] (Fed. Cir. 1991). Nevertheless, the Board refuted the three methods disclosed in Noelle's specification and endorsed by Clark. First, the Board found that the immunization technique found in the prior art would be ineffective because, at the relevant time, one skilled in the art would not have had a reasonable expectation of success of identifying the activated T-cells that produced the required CD40CR antigen or of isolating the antigen itself. Second, the Board found that it would have been "extremely difficult"for a person of ordinary skill in the art to isolate successfully CD40-Ig, which, as Noelle asserted, could then be used to obtain the claimed CD40CR antibodies. Third, the Board cited statements made during the prosecution of Armitage application 07/969,703 for the proposition that a skilled artisan could not have used expression cloning to isolate CD40CR antibody with a reasonable likelihood of success.

Thus, the Board determined that a person of ordinary skill in the art would not have been reasonably likely to isolate human CD40CR antibody given Noelle's claimed invention of mouse CD40CR antibody. As a result, the Board found no interference-in-fact between Noelle's remaining murine CD40CR antibody claim and Lederman's claim

to the human form of CD40CR antibody. Noelle timely appealed to this court and we have jurisdiction under 28 U.S.C. §1295(a)(4)(A) (2000).

DISCUSSION

Whether a specification complies with the written description requirement of 35 U.S.C. §112, paragraph (1), is a question of fact, *Vas-Cath Inc. v. Mahurkar*, 935 F.2d 1555, 1562 [19 USPQ2d 1111] (Fed. Cir. 1991), and is, in appeals from the Board, reviewed under the substantial evidence standard. *In re Gartside*, 203 F.3d 1305, 1315 [53 USPQ2d 1769] (Fed. Cir. 2000). To apply a substantial evidence standard, this court must "examin[e] the record as a whole, taking into account evidence that both justifies and detracts from an agency's decision." *Id.* at 1312. A reviewing court must ask "whether a reasonable fact finder could have arrived at the agency's decision." *Id.* "[T]he possibility of drawing two inconsistent conclusions from the evidence does not prevent an administrative agency's finding from being supported by substantial evidence." *Id.*

A. *Entitlement to Priority*

The written description requirement has been defined many times by this court, but perhaps most clearly in *Vas-Cath*. The court held as follows:

> 35 U.S.C. §112, first paragraph, requires a "written description of the invention" which is separate and distinct from the enablement requirement. The purpose of the "written description"requirement is broader than to merely explain how to "make and use"; the applicant must also convey with reasonable clarity to those skilled in the art that, as of the filing date sought, he or she was in possession *of the invention*. The invention is, for purposes of the "written description" inquiry, *whatever is now claimed. Vas-Cath*, 935 F.2d at 1563-64 (emphasis in original). Thus, the test to determine if an application is to receive the benefit of an earlier filed application is whether a person of ordinary skill in the art would recognize that the applicant possessed what is claimed in the later filed application as of the filing date of the earlier filed application. An earlier application that describes later-claimed genetic material only by a statement of function or result may be insufficient to meet the written description requirement. *See Regents*, 119 F.3d at 1566. This court has held that a description of DNA "'requires a precise definition, such as by structure, formula, chemical name, or physical properties,' not a mere wish or plan for obtaining the claimed chemical invention." *Id.* (quoting *Fiers v. Revel*, 984 F.2d 1164, 1170 [25 USPQ2d 1601] (Fed. Cir. 1993)). Therefore, this court has held that statements in the specification describing the functional characteristics of a DNA molecule or methods of its isolation do not adequately describe a particular claimed DNA sequence. Instead "an adequate written description of DNA requires more than a mere statement that it is part of the invention and reference to a potential method for isolating it; what is required is a description of the DNA itself." *Id.* at 1566-67 (quoting *Fiers*, 984 F.2d at 1171). It should be noted, however, that this court in *Vas-Cath*

> warned that each case involving the issue of written description, "must be decided on its own facts. Thus, the precedential value of cases in this area is extremely limited." *Vas-Cath*, 935 F.2d at 1562 (quoting *In re Driscoll*, 562 F.2d 1245, 1250 [195 USPQ 434] (C.C.P.A. 1977)).

Indeed, the court in *Enzo Biochem v. Gen-Probe, Inc.*, 323 F.3d 956, 964 (Fed. Cir. 2002) ("*Enzo Biochem II*"), stated that "the written description requirement would be met for all of the claims [of the patent at issue] if the functional characteristic of [the claimed invention was] coupled with a disclosed correlation between that function and a structure that is sufficiently known or disclosed." Also, the court held that one might comply with the written description requirement by depositing the biological material with a public depository such as the American Type Culture Collection ("ATCC"). *Id.* at 970. The court proffered an example of an invention successfully described by its functional characteristics. The court stated:

> For example, the PTO would find compliance with 112, paragraph 1, for a claim to an isolated antibody capable of binding to antigen X, notwithstanding the functional definition of the antibody, in light of the well defined structural characteristics for the five classes of antibody, the functional characteristics of antibody binding, and the fact that the antibody technology is well developed and mature. *Id.* The court adopted the USPTO Guidelines as persuasive authority for the proposition that a claim directed to "any antibody which is capable of binding to antigen X" would have sufficient support in a written description that

> disclosed "*fully characterized* antigens." Synopsis of Application of Written Description Guidelines, at 60, *available at* http://www.uspto.gov/web/menu/written.pdf (last visited Jan. 16, 2003) (emphasis added).

[1] Therefore, based on our past precedent, as long as an applicant has disclosed a "*fully characterized* antigen,"either by its structure, formula, chemical name, or physical properties, or by depositing the protein in a public depository, the applicant can then claim an antibody by its binding affinity to that described antigen.

[2] Noelle did not provide sufficient support for the claims to the human CD40CR antibody in his '480 application because Noelle failed to disclose the structural elements of human CD40CR antibody or antigen in his earlier '799 application. Noelle argues that because antibodies are defined by their binding affinity to antigens, not their physical structure, he sufficiently described human CD40CR antibody by stating that it binds to human CD40CR antigen. Noelle cites *Enzo Biochem II* for this proposition. This argument fails, however, because Noelle did not sufficiently describe the human CD40CR antigen at the time of the filing of the '799 patent application. In fact, Noelle only described the mouse antigen when he claimed the mouse, human, and genus forms of CD40CR antibodies by citing to the ATCC number of the hybridoma secreting the mouse CD40CR antibody. If Noelle had sufficiently described the human form of CD40CR antigen, he

could have claimed its antibody by simply stating its binding affinity for the "fully characterized" antigen. Noelle did not describe human CD40CR antigen. Therefore, Noelle attempted to define an unknown by its binding affinity to another unknown. As a result, Noelle's claims to human forms of CD40CR antibody found in his '480 application cannot gain the benefit of the earlier filing date of his '799 patent application.

Moreover, Noelle cannot claim the genus form of CD40CR antibody by simply describing mouse CD40CR antigen. Noelle cites *Staehelin v. Secher*, 24 U.S.P.Q.2d 1513, 1519 (Bd. Pat. App. & Int. Sept. 28, 1992), as support for his argument that he has rights to the genus form of CD40CR antibody. In *Staehelin*, Dr. Secher had developed a hybridoma that produced a monoclonal antibody targeted to an antigen unavailable in pure form. *Id.* The antigen was human leukocyte interferon. *Id.* In Secher's foreign application, he had reported the isolation of a hybridoma-secreting antibody to human leukocyte interferon. *Id.* In his subsequent U.S. application, Secher claimed the genus form of the antibody. *Id.* at 1520. The Board held, "Secher's disclosure ... would have reasonably conveyed to a person possessing ordinary skill in the art that Secher possessed the genus later claimed by them in their U.S. application in the sense of 35 U.S.C. 112, first paragraph." *Id.* The Board held it is not necessary to describe the exact details for preparing every species within the genus in order to claim the genus. *Id.* (citing *Utter v. Hiraga*, 845 F.2d 993, 998 [6 USPQ2d 1709] (Fed. Cir. 1988)). Thus, Noelle argues, the disclosure in his previous '799 application of the mouse form of CD40CR antibody was sufficient to support his later genus claims.

Noelle's reliance on *Staehelin* is misplaced. First, it is a decision from the Board of Patent Appeals and Interferences which may be persuasive but it is not binding precedent on this court. Second, the Board in *Staehelin* cited *Utter* to support the proposition that a patentee need not cite every species of an antibody in order to claim the genus of that antibody. In *Utter*, this court held that not every species of scroll compressor used in air conditioners must be described in order for a genus claim to meet the written description requirement. 845 F.2d at 994. Since the Board's decision in *Staehelin*, this court has subsequently held that a patentee of a biotechnological invention cannot necessarily claim a genus after only describing a limited number of species because there may be unpredictability in the results obtained from species other than those specifically enumerated. *See Enzo Biochem II*, 323 F.3d at 965; *Regents*, 119 F.3d at 1568. Therefore, to the extent the Board's decision in *Staehelin* conflicts with our decisions in *Enzo Biochem II* and *Regents*, it has been limited in applicability.

The Board was also correct in its determination that the human and genus claims were anticipated by Lederman '771 and Armitage '974. The Board's decision was supported by substantial evidence, and Noelle conceded that without the earlier filing date of his '799 application, his claims were indistinguishable from the prior art cited by the Board.

B. *Interference-In-Fact*

Interference proceedings are subjected to the requirements of 37 C.F.R. §§1.601 – 1.690 (2003), promulgated pursuant to 35 U.S.C. §135(a). *Eli Lilly v. Bd. of Regents of the Univ. of Wash.*, 334 F.3d 1264, 1267 [67 USPQ2d 1161] (Fed. Cir. 2003). A patent interference is designed to "determine whether two patent applications (or a patent application and an issued patent) are drawn to the same 'patentable invention' and, if so, which of the competing parties was first to invent the duplicative subject matter." *Id.* (citing *Conservolite, Inc. v. Widmayer*, 21 F.3d 1098, 1100-01 [30 USPQ2d 1626] (Fed. Cir. 1994)); *see also* 37 C.F.R §1.601(j).6 In order to determine whether the two parties claim the same patentable invention, the USPTO has promulgated a "two-way" test, which has been approved by this court. *Eli Lilly*, 334 F.3d at 1270. The two-way test reads as follows:

> Invention "A" is the same patentable invention as an invention "B" when invention "A" is the same as (35 U.S.C. 102) or is obvious (35 U.S.C. 103) in view of invention "B"assuming invention "B" is prior art with respect to invention "A". Invention "A" is a separate patentable invention with respect to invention "B" when invention "A" is new (35 U.S.C. 102) and non-obvious (35 U.S.C. 103) in view of invention "B"assuming invention "B" is prior art with respect to invention "A". 37 C.F.R. §1.601(n). In order for an interference-in-fact to exist, invention A must anticipate or make obvious invention B, *and* invention B must anticipate or make obvious invention A, thereby meeting both prongs of the "two-way" test. *Eli Lilly*, 334 F.3d at 1268; *accord Winter v. Fujita*, 53 U.S.P.Q.2d 1234, 1243 (Bd. Pat. App. & Int. Nov. 16, 1999). The Board in the present case worded the two-way test in a different way as follows: Thus, for Lederman to succeed in its motion for no interference-in-fact, Lederman need only demonstrate that: (i) Lederman's claims are not anticipated or rendered obvious by Noelle's remaining "mouse" claims; *or* (ii) Noelle's remaining "mouse" claims are not anticipated or rendered obvious by Lederman's claims. (Emphasis in original).

[3] Noelle's argument that the Board improperly required a two-way patentability test, or, as the Board phrased it, a "one-way distinctiveness" test, is without merit in light of this court's recent ruling in *Eli Lilly* upholding the Director's two-way test as consistent with the language of the regulation. 334 F.3d at 1268. Therefore, the Board applied the proper "two-way test." First, it determined that "one skilled in the art lacked a reasonable expectation of success of obtaining Lederman's claimed 'human' subject matter when provided with Noelle's 'mouse' subject matter and using the screening techniques cited by Noelle." Although the Board did not have to conduct the second prong of the test to find no interference-in-fact, it did so anyway by finding that "one skilled in the art would have lacked a reasonable expectation of success of obtaining Noelle's 'mouse'subject matter when provided with Lederman's claimed 'human'subject matter and using the same screening methods." Therefore, the Board utilized the correct test to find no interference-in-fact.

Noelle's argument that the Board erred in its application of the obviousness question in the interference-in-fact analysis by ignoring the specification in Noelle's '480 application is also without merit. Both Lederman and Noelle concede that the anticipation

portion of the interference-in-fact analysis is not an issue in light of the agreed variance between claims to mouse versus human forms of CD40CR antibodies. Thus, only the obviousness analysis pursuant to 35 U.S.C. §103 is left to be determined. Obviousness is determined as follows:

> a proper analysis under §103 requires, *inter alia*, consideration of two factors: (1) whether the prior art would have suggested to those of ordinary skill in the art that they should make the claimed composition or device, or carry out the claimed process; and (2) whether the prior art would also have revealed that in so making or carrying out, those of ordinary skill would have a reasonable expectation of success. *In re Vaeck*, 947 F.2d at 493. Both the suggestion and the reasonable expectation of success "must be founded in the prior art, not in the

> applicant's disclosure." *Id.*; *see also In re Dow Chem. Co.*, 837 F.2d 469, 473 [5 USPQ2d 1529] (Fed. Cir. 1988).

The parties agree that a skilled artisan would have been motivated to obtain the human CD40CR antibody if the mouse CD40CR antibody were available. The two parties disagree, however, as to whether the prior art would provide a reasonable likelihood of success in so doing. Therefore, the issue before us is whether substantial evidence supports the Board's determination that one of ordinary skill in the art would not have had a reasonable expectation of success of isolating the other party's invention given the disclosures found in the claims. A reasonable likelihood of success does not necessarily mean an absolute predictability, but rather a reasonable expectation of success. *Yamanouchi Pharm. v. Danbury Pharmacal, Inc.*, 231 F.3d 1339, 1343 [56 USPQ2d 1641] (Fed. Cir. 2000).

Noelle argues that the methods disclosed in his '799 patent application would have provided a reasonable likelihood of success for a person of ordinary skill in the art to isolate human CD40CR antibodies using mouse CD40CR antibodies. Specifically, Noelle argues it would have been obvious to a skilled artisan to use the CD40-Ig fusion protein disclosed in the '799 application as a screen to locate, within a hybridomal library, monoclonal antibodies that specifically bind to human CD40CR antigen. Noelle further argues the Board improperly ignored this method of antibody isolation merely because it was disclosed in Noelle's written description as opposed to Noelle's claims.

[4] The Board correctly found no interference-in-fact between Noelle's claims and Lederman's claims. First, the Board was correct in not considering Noelle's methods of isolation of human CD40CR antigen using CD40-Ig found in his '799 specification because the methods were neither part of the parties' inventions nor "prior art." USPTO rules establish that an interference-in-fact exists when both parties claim the "same patentable invention." 37 C.F.R. §1.601(n). A patentee's invention is only found in a patentee's claims, unless the patentee uses sufficient means-plus-function language to invoke 35 U.S.C. §112, paragraph (6). Thus, if the Board is to compare two inventions,

the Board must only compare the parties' claims. Noelle does not claim a method of isolating CD40CR antigens, CD40-Ig, or the receptor CD40 itself. Obviously, if certain terms in Noelle's or Lederman's claims were ambiguous, we could resort to the specification or other sources to define those terms; however, it is unnecessary here as none of the terms in the claims are ambiguous. Therefore, Noelle cannot rely on a method of isolating human CD40CR antigen using CD40-Ig in order to prove obviousness between his invention and Lederman's invention because the method is not claimed.

Second, the Board's determination was supported by substantial evidence because a person of ordinary skill in the art, given the state of prior art at the time of the '799 filing, would not have had a reasonable likelihood of success in isolating human CD40CR antibodies from the mouse CD40CR antigen and its antibody. Noelle argues that one skilled in the art would have had a reasonable likelihood of success in manufacturing a set of hybridomas that secrete monoclonal antibodies to activated human helper T-cell surface antigens. Noelle, as outlined previously, cited three different screening methods disclosed in his specification that would isolate the desired hybridomas and their antibodies. The first two of Noelle's proposed screening methods require the use of CD40Ig. As the expert testimony of Dr. Aruffo, the named inventor in the patent claiming CD40-Ig, indicated to the Board, it would have been unpredictable and unreasonable to expect a skilled artisan to produce CD40-Ig given the state of the art at the time.

Finally, Noelle's expert witness, Dr. Clark, addressed the third and final proposed screening method. Dr. Clark declared that, given the mouse form of CD40CR antibody or CD40-Ig and the utilization of expression cloning methods available at the time, a person of ordinary skill in the art would have had a reasonable expectation of success in isolating the human form of CD40CR antigen. Armitage, however, during the prosecution of his '703 application, stated that the use of expression cloning could not have reasonably led to successful isolation of human CD40CR antigen.

After examining the record as a whole, we conclude there was substantial evidence to support the Board's decision. The Board's decision was reasonable in that, given the state of the art in the early 1990s as described by the expert witnesses, a person of ordinary skill in the art would not have had a reasonable likelihood of success in isolating human

CD40CR antigen given mouse CD40CR antigen.

CONCLUSION

For the foregoing reasons, the decision of the Board rejecting claims 51, 52, 53, 56, 59, and 60 of Noelle's U.S. Application No. 08/742,480 is affirmed. The decision of the Board granting Lederman's preliminary motion of no interference-in-fact is also affirmed.

AFFIRMED

No costs.

Footnotes

1 For additional background on the function of antibodies, as well as methods of isolating antibodies, *see In re Wands*, 858 F.2d 731, 733-34 [8 USPQ2d 1400] (Fed. Cir. 1988) and *Hybritech Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1368-69 [231 USPQ 81] (Fed. Cir. 1986).

2 Cell differentiation is the process of modifying a cell's structure and function in order for it to become more specialized and specific to the invading antigen.

3 CD40CR antigen is also referred to as "CD40 counter receptor," "CD40 ligand," "CD40L," and simply "CD40CR." Lederman uses the term "5c8 antigen" or "T-B cell-activating molecule"("T-BAM") to designate the 30-kilodalton human form of CD40CR antigen. Noelle uses the term "gp39" (glycoprotein 39 kD) to describe the 39-kilodalton mouse form of CD40CR antigen.

4 A hybridoma is a man-made tissue culture consisting of cancerous B-cells fused to B-cells producing the antibody of choice. A hybridoma produces unlimited amounts of a desired "monoclonal" antibody. *See Hybritech*, 802 F.2d at 1368-69 (explaining the method for creating and using hybridomas).

5 CD40-Ig is a fusion protein wherein a portion of the CD40 receptor is fused to an immunoglobulin (Ig). CD40-Ig is therefore not expressed on the surface of a B-cell but rather is essentially a soluble, free-floating molecule.

6 37 C.F.R. §1.601 (j) reads as follows:

An interference-in-fact exists when at least one claim of a party that is designated to correspond to a count and at least one claim of an opponent that is designated to correspond to the count define the same patentable invention.

- End of Case -

In re Wallach, 71 USPQ2d 1939 (CA FC 2004)

71 USPQ2D 1939
In re Wallach

U.S. Court of Appeals Federal Circuit

No. 03-1327
Decided August 11, 2004

Headnotes

PATENTS

[1] Patentability/Validity — Specification — Written description (§115.1103)

State of molecular biology art has developed such that complete amino acid sequence

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of particular protein may put inventor in possession of genus of DNA sequences encoding it, and person of ordinary skill in art, as of filing date for application claiming isolated DNA molecules that encode particular proteins, may therefore have been in possession of entire genus of DNA sequences that can encode disclosed partial protein sequence, even if individual species within that genus might not have been described or rendered obvious; moreover, applicant is not required to list every possible permutation of nucleic acid sequences that can encode particular protein for which amino acid sequence is disclosed, since it is routine matter to convert back and forth between amino acid sequence and sequences of nucleic acid molecules that can encode it.

[2] Patentability/Validity — Specification — Written description (§115.1103)

Claims in application directed to isolated DNA molecules encoding proteins that inhibit cytotoxic effects of tumor necrosis factor were properly rejected for failure to satisfy written description requirement of 35 U.S.C. §112, since applicants claimed nucleic acids encoding protein for which they provided only partial sequence, and without approximately 95 percent of amino acid sequence that applicants did not disclose, it cannot be held that DNA molecules claimed in application have been described, since applicants' contention that they were in physical possession of protein does not establish

their knowledge of that protein's amino acid sequence or any of its other descriptive properties, even though amino acid sequence is inherent property of protein, and since application does not provide adequate functional description, in that, with only partial amino acid sequence disclosed, chemical structure of nucleic acid molecules that can serve function of encoding protein's amino acid sequence cannot be determined.

Case History and Disposition
Appeal from the U.S. Patent and Trademark Office, Board of Patent Appeals and Interferences.

Patent application (serial no. 08/485,129) of David Wallach, Hartmut Engelmann, Dan Aderka, Daniela Novick, and Menachem Rubinstein. Applicants appeal from decision upholding rejection of claims in application for failure to satisfy written description requirement of 35 U.S.C. §112. Affirmed.

Attorneys:
Roger L. Browdy, of Browdy and Neimark, Washington, D.C., for appellants.

Mary L. Kelly, associate solicitor; John M. Whealan, solicitor; Raymond T. Chen, Stephen Walsh, and William LaMarca, associate solicitors, U.S. Patent and Trademark Office, Arlington, Va., for the PTO.

Judge:
Before Mayer, chief judge, and Lourie and Gajarsa, circuit judges.

Opinion Text

Opinion By:
Lourie, J.

David Wallach, Hartmut Engelmann, Dan Aderka, Daniela Novick, and Menachem Rubinstein (collectively, "Appellants") appeal from the decision of the United States Patent and Trademark Office ("PTO") Board of Patent Appeals and Interferences affirming the rejection of claims 11-13, 35-38, 43, 44, 46-49, 51-54, 56-61, 63, and 64 of United States patent application 08/485,129 under the written description requirement of 35 U.S.C. §112. *In re Wallach*, Appeal No. 2002-1363 (Bd. Pat. Apps. & Interfs. Dec. 26, 2002). We affirm.

BACKGROUND

In the 1980s, Appellants apparently discovered two specific proteins isolated from human urine that, among other things, selectively inhibit the cytotoxic effect of tumor necrosis factor ("TNF"). They named the compounds TNF binding proteins I & II ("TBP-I" and "TBP-II"). After obtaining a partial amino acid sequence of the N-terminal portion of TBP-II and determining that the complete protein has a molecular weight of about 30 kilodaltons ("kDa") when measured by sodium dodecyl sulfate polyacrylamide gel electrophoresis ("SDS-PAGE") under reducing conditions, Appellants filed a patent application including, *inter alia*, claims directed to proteins having that molecular weight and partial sequence (*i.e.*, threonine-proline-tyrosine-alanine-proline-glutamic acid-proline-glycine-serine-threonine, or "Thr-Pro-Tyr-Ala-Pro-Glu-Pro-Gly-Ser-Thr") and having the ability to inhibit the cytotoxic effect of TNF. Appellants' application also included claims to isolated DNA molecules that encode the claimed proteins. The PTO issued a restriction requirement and Appellants filed divisional applications. The claims directed to the proteins having the stated partial sequence are currently involved in an interference proceeding and are not at issue here. The claims

at issue, those directed to the DNA, were rejected under §112 "as based on a specification which does not provide an adequate written description of the claimed invention." *Wallach*, slip op. at 2. After several unsuccessful attempts to traverse that rejection, Appellants appealed to the Board.

Citing this court's decisions in *Amgen, Inc. v. Chugai Pharmaceutical Co.*, 927 F.2d 1200 (Fed. Cir. 1991), *Fiers v. Revel*, 984 F.2d 1164 [25 USPQ2d 1601] (Fed. Cir. 1993), and *Regents of the University of California v. Eli Lilly & Co.*, 119 F.3d 1559 [43 USPQ2d 1398] (Fed. Cir. 1997), the Board affirmed the examiner's rejection. In particular, the Board held that "(1) applicants do not describe the genetic material sought to be patented in claim 11 with sufficient specificity in their specification; and (2) the examiner did not err in finding that claim 11 is based on a specification which does not provide adequate, written descriptive support for the claimed subject matter." *Wallach*, slip op. at 8-9.*

Appellants now appeal. We have jurisdiction pursuant to 28 U.S.C. § 1295(a)(4)(A).

DISCUSSION

Claim 11 of the '129 application reads as follows:

> 11. An isolated DNA molecule comprising a contiguous nucleotide sequence coding for a protein consisting of naturally occurring human Tumor Necrosis Factor (TNF) Binding Protein II, herein designated TBP-II, said TBP-II including the amino acid sequence: Thr-Pro-Tyr-Ala-Pro-Glu-Pro-Gly-Ser-Thr in the portion of the protein sequenced by N-terminal sequence analysis, said protein having the ability to inhibit the cytotoxic effect of TNF, wherein said naturally occurring TBP-II protein is the same as that protein having the ability to inhibit the cytotoxic effect of TNF which, after being purified by subjecting a crude protein recovered from a dialyzed concentrate of human urine to affinity chromatography on a column of immobilized TNF, elutes from a reversed-phase high pressure liquid chromatography column as a single peak in a fraction corresponding to about 31% acetonitrile and shows a molecular weight of about 30 kDa when measured by SDS-PAGE under reducing conditions.

On appeal, Appellants argue that the PTO has effectively conceded that the TBP-II protein, which the claimed isolated DNA encodes, is sufficiently described in the specification to comply with § 112, because the claims of United States patent application 07/930,443, of which the '129 application is a division (which, by definition, has the same specification), have been allowed but for their involvement in an interference proceeding. According to Appellants, those claims do not differ in substance from the present claims except insofar as they are directed to a partial protein sequence, rather than to the DNA sequences encoding the protein. Appellants contend that that is not a meaningful distinction, because the genetic code is based on an unequivocal correspondence between amino acids and encoding DNA codons, and determination of the amino acid sequence of a protein automatically puts one in possession of all DNA sequences encoding that protein. Appellants also argue that the complete amino acid sequence of a protein is an inherent property of an isolated protein that has been fully characterized by partial amino acid sequence and other characteristics, and that the complete amino acid sequence of a protein therefore puts one in possession of all DNA sequences encoding it. Therefore, according to Appellants, the specification establishes that the present inventors were in fact in possession of the entire claimed genus of DNA sequences at the time the application was filed.

Appellants also argue that this case is distinguishable from past written description cases such as *Amgen v. Chugai* and *Fiers*, because Appellants have provided an actual amino acid sequence that is encoded by the claimed DNA, not simply the name of the protein and a statement that the DNA can be obtained by reverse transcription. Appellants contend that this case is also distinguishable from *Lilly* because the inventors here are not attempting to claim DNA molecules encoding a plurality of unknown proteins from various species having no common features, but only those encoding the single protein sequence that is actually set forth in the specification.

Finally, Appellants argue that, because there is a known correlation between the function (*i.e.*, encoding a specified amino acid sequence) and structure, this is the quintessential example of the sort of functional description permitted by §112 in view of our decision in *Enzo Biochem, Inc. v. Gen-Probe Inc.*, 296 F.3d 1316 (Fed. Cir. 2002). Appellants argue that our recent decision in *Amgen Inc. v. Hoechst Marion Roussel, Inc.*, 314 F.3d 1313 [65 USPQ2d 1385] (Fed. Cir. 2003), which issued after the Board's opinion in the present case, reaffirmed that §112 only requires a court to determine whether a specification conveys to one of ordinary skill in the art as of the filing date that the inventors invented the claimed subject matter.

The PTO responds by arguing that Appellants' specification includes neither any actual DNA sequence within the scope of the claims nor the complete amino acid sequence of the TBP-II protein, but only the sequence of ten out of the 185-192 amino acids that make up the protein. Furthermore, the PTO argues, the only disclosed function of the claimed DNA molecules is to encode the TBP-II protein, and no information is provided from which the claimed DNA molecules can be distinguished from other DNA molecules. According to the PTO, the identity of the nucleic acid encoding a protein is not an inherent property of the protein. If Appellants' reasoning were accepted, the PTO asserts, the result would be that the disclosure of an isolated protein would be prior art under §102 with respect to claims directed to any nucleic acid encoding the protein. Finally, the PTO contends, substantial evidence supports the Board's factual finding that Appellants'specification does not adequately describe the claimed genus of DNA molecules.

[1] As a preliminary matter, we agree with Appellants that the state of the art has developed such that the complete amino acid sequence of a protein may put one in possession of the genus of DNA sequences encoding it, and that one of ordinary skill in the art at the time the '129 application was filed may have therefore been in possession of the entire genus of DNA sequences that can encode the disclosed partial protein sequence, even if individual species within that genus might not have been described or rendered obvious. *Cf. In re Deuel*, 51 F.3d 1552 [34 USPQ2d 1210] (Fed. Cir. 1995). Thus, for example, the RNA molecules required to encode the described amino acid sequence must necessarily have the following sequence: ACN-CCN-UAY-GCN-CCN-GAR-CCN-GGN-(UCN or AGY)-ACN, where N is A, G, C, or U; Y is U or C; and R is G or A. *See* James D. Watson et al., *Molecular Biology of the Gene* 356-57 (3d ed. 1977), *cited in* '129 application. A claim to the genus of DNA molecules complementary to the RNA having the sequences encompassed by that formula, even if defined only in terms of the protein sequence that the DNA molecules encode, while containing a large number of species, is definite in scope and provides the public notice required of patent applicants. Indeed, the PTO's Manual of Patent Examining Procedure ("MPEP") states:

> Description of a representative number of species does not require the description to be of such specificity that it would provide individual support for each species that the genus embraces. For example, in the molecular biology arts, if an applicant disclosed an amino acid sequence, it would be unnecessary to provide an explicit disclosure of nucleic acid sequences that encoded the amino acid sequence. Since the genetic code is widely known, a disclosure of an amino acid sequence would provide sufficient information such that one would accept that an applicant was in possession of the full genus of nucleic acids encoding a given amino acid sequence, but not necessarily any particular species. MPEP §2163.II.A.3.a.ii. (8th ed., rev. 2 2001).

Moreover, we see no reason to require a patent applicant to list every possible permutation of the nucleic acid sequences that can encode a particular protein for which the amino acid sequence is disclosed, given the fact that it is, as explained above, a routine matter to convert back and forth between an amino acid sequence and the sequences of the nucleic acid molecules that can encode it.

[2] Nonetheless, Appellants did not claim the nucleic acid molecules that encode the simple protein sequence that they disclosed. Rather, they claimed the nucleic acids encoding a protein for which they provided only a partial sequence. Appellants concede that it is now known that urinary TBP-II has a sequence of 185-192 amino acids. Without the approximately 95% of the amino acid sequence

that Appellants did not disclose, we cannot say that the DNA molecules claimed in the '129 application have been described. As the MPEP explains, "disclosure of a partial structure without additional characterization of the product may not be sufficient to evidence possession of the claimed invention." MPEP §2163.II.A.3.a.i. The Board's decision was thus consistent with its guidance in the MPEP. Here, Appellants disclosed a partial structure and possibly sufficient additional characterization of the TBP-II protein to satisfy the PTO that they were in possession of the claimed subject matter in their '443 application, but that additional characterization contributes little, if anything, to the description of the DNA molecules claimed in the '129 application.

Appellants argue that "[a]s appellants have demonstrated possession of the TBP-II protein, appellants were also necessarily in possession of its inherent amino acid sequence, as well as all of the DNA sequences encoding that amino acid sequence." We disagree. Whether Appellants were in possession of the protein says nothing about whether they were in possession of the protein's amino acid sequence. Although Appellants correctly point out that a protein's amino acid sequence is an inherent property of the protein, the fact that Appellants may have isolated and thus physically possessed TBP-II does not amount to knowledge of that protein's sequence or possession of any of its other descriptive properties. Appellants have not provided any evidence that the full amino acid sequence of a protein can be deduced from a partial sequence and the limited additional physical characteristics that they have identified. Without that full sequence, we cannot agree with Appellants that they were possession of the claimed nucleic acid sequences. In *Amgen v. Chugai*, we explained that:

> A gene is a chemical compound, albeit a complex one, and it is well established in our law that conception of a chemical compound requires that the inventor be able to define it so as to distinguish it from other materials, and to describe how to obtain it. Conception does not occur unless one has a mental picture of the structure of the chemical, or is able to define it by its method of preparation, its physical or chemical properties, or whatever characteristics sufficiently distinguish it. It is not sufficient to define it solely by its principal biological property, ... because an alleged conception having no more specificity than that is simply a wish to know the identity of any material with that biological property. 927 F.2d at 1206. Until Appellants obtained the complete amino acid sequence of TBP-II, they had no more than a wish to know the identity of the DNA encoding it.

As Appellants point out, we have recognized that the written description requirement can in some cases be satisfied by functional description. *See, e.g.*, *Enzo*, 296 F.3d at 1324 ("It is not correct, however, that all functional descriptions of genetic material fail to meet the written description requirement."). Nonetheless, such functional description can be sufficient only if there is also a structure-function relationship known to those of ordinary skill in the art. As we explained above, such a well-known relationship exists between a nucleic acid molecule's structure and its function in encoding a particular amino acid sequence: Given the amino acid sequence, one can determine the chemical structure of all nucleic acid molecules that can serve the function of encoding that sequence. Without that sequence, however, or with only a partial sequence, those structures cannot be determined and the written description requirement is consequently not met. As we explained in *Enzo*, the *Guidelines for Examination of Patent Applications under the 35 U.S.C. §112, ¶ 1, "Written Description" Requirement*, 66 Fed. Reg. 1099 (Jan. 5, 2001) ("*Guidelines*"), state that

> the written description requirement can be met by "show[ing] that an invention is complete by

disclosure of sufficiently detailed, relevant identifying characteristics ... *i.e.*, complete or partial structure, other physical and/or chemical properties, *functional characteristics when coupled with a known or disclosed correlation between function and structure*, or some combination of such characteristics." *Guidelines*, 66 Fed. Reg. at 1106 (emphasis added). *Enzo*, 296 F.3d at 1324-25 (emphasis added). Appellants have provided no evidence that there is any known or disclosed correlation between the combination of a partial structure of a protein, the protein's biological activity, and the protein's molecular weight, on the one hand, and the structure of the DNA encoding the protein on the other.

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CONCLUSION

The Board correctly affirmed the examiner's determination that the specification of the '129 application does not provide an adequate written description of the pending claims. Accordingly, the Board's decision is

AFFIRMED.

Footnotes

* The Board treated all of the appealed claims as standing or falling together with claim 11, pursuant to 37 C.F.R. §1.192(c)(7), and decided the appeal on the basis of that claim alone. *Wallach*, slip op. at 5. Appellants do not challenge the Board on that point, and we likewise decide this appeal only on the basis of that claim.

- End of Case -